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14. ABSTRACT: The goal of this project was to design new chemical tools to selectively probe the molecular mechanisms of action of rapid estrogen receptor action and their relevance to breast cancer drugs like tamoxifen. Over the course of the project, we synthesized and tested approximately 15 new estrogen receptor modulators, some with novel activity in terms of both classic transcriptional and rapid response modulation. We discovered that the structure activity relationship for some rapid estrogen responses is different than the SAR required for transcriptional regulation, but that rapid responses could not be separated from the transcriptional modulation. We synthesized a novel metabolite of tamoxifen, called endoxifen, which appears to be the major bioactive metabolite of tamoxifen in women. This work also resulted in a letter to the FDA concerning possible drug interactions between tamoxifen and women taking certain antidepressants. We also made a new polymer-based conjugate of 4-hydroxytamoxifen that not only shows great uptake into ER positive breast cancer cells, but also shows antiproliferative activity against antiestrogen-sensitive and antiestrogen-resistant breast cancer cell lines. We are currently trying to translate this unexpected finding into the design of new experimental therapeutics for the treatment of tamoxifen-resistant breast cancer, an urgent need for breast cancer patients.

15. SUBJECT TERMS
estrogen, estrogen receptor, nongenomic signaling, SERMs, tamoxifen, raloxifene, antiestrogen resistance, drug discovery

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Introduction

Estrogens and antiestrogens are of utmost importance in the development, treatment and possible chemoprevention of breast cancer. Although much progress has been made in understanding the mechanisms by which estrogen and SERMs function, a class of mechanisms that is getting increased scrutiny is the so-called “non-genomic” response that is due to modulation of cell signaling pathways other than direct transcriptional regulation. There is much debate as to the receptors responsible for these responses and the mechanisms by which they operate. This proposal aims to design and use selective chemical probes to begin to answer those questions. In particular, estrogen responses related to breast cell proliferation and resistance to apoptosis will be studied using a variety of chemical probes including polymer-based drugs designed to test the potential role of cell surface estrogen receptors.

Body

This project has focused on developing and testing chemical probes of rapid responses to estrogen relevant to the treatment and chemoprevention of breast cancer. The proposed project was broken into 4 main tasks:

1. Determine the effects of the ligand structure on both rapid signaling and estrogen receptor-mediated transcription by testing a screening library of various known and novel estrogen response modulators in a number of assays.
2. Test the potential role of estrogen receptor (ER) alpha or ER beta in rapid signaling, by performing assays with various N-terminal deletion and chimera mutants of ER alpha and ER beta.
3. Test the role of cell surface receptors in rapid estrogen signaling, by developing cell-impermeable, non-proteinaceous estradiol conjugates.
4. Test the potential role of rapid estrogen signaling in breast cancer proliferation and survival, by treating various breast cell lines with selective compounds discovered above and measuring changes in cell growth, cytotoxicity and apoptosis

As will be described in the rest of this report, much significant progress was made with task 1, 3 and 4 while only some progress in task 2 was made due to some difficulties encountered in assay development and in some of the findings discovered in tasks 1 and 3. As will be described later in the report, it is clear from our on this project that it is very difficult to purely separate these “rapid” responses from other downstream effects of receptor action and that the most appropriate approach is one that investigates the integration of rapid responses arising from receptor crosstalk with other downstream events. This has important implications in ligand design of hormone-based breast cancer treatments. A summary on the research follows and is organized by the specific tasks of the statement of work.

Task 1. Determine the effects of the ligand structure on both rapid signaling and estrogen receptor-mediated transcription by testing a screening library of various known and novel estrogen response modulators in a number of assays.

<u>Task 1a.</u> Generate the screening library by synthesizing a small number of estradiol and triphenylethylethylene analogs and combining it with commercially available and previously synthesized compounds.
The initial screening proposed is shown in **Figure 1** includes a few compounds that were not in the initial panel.

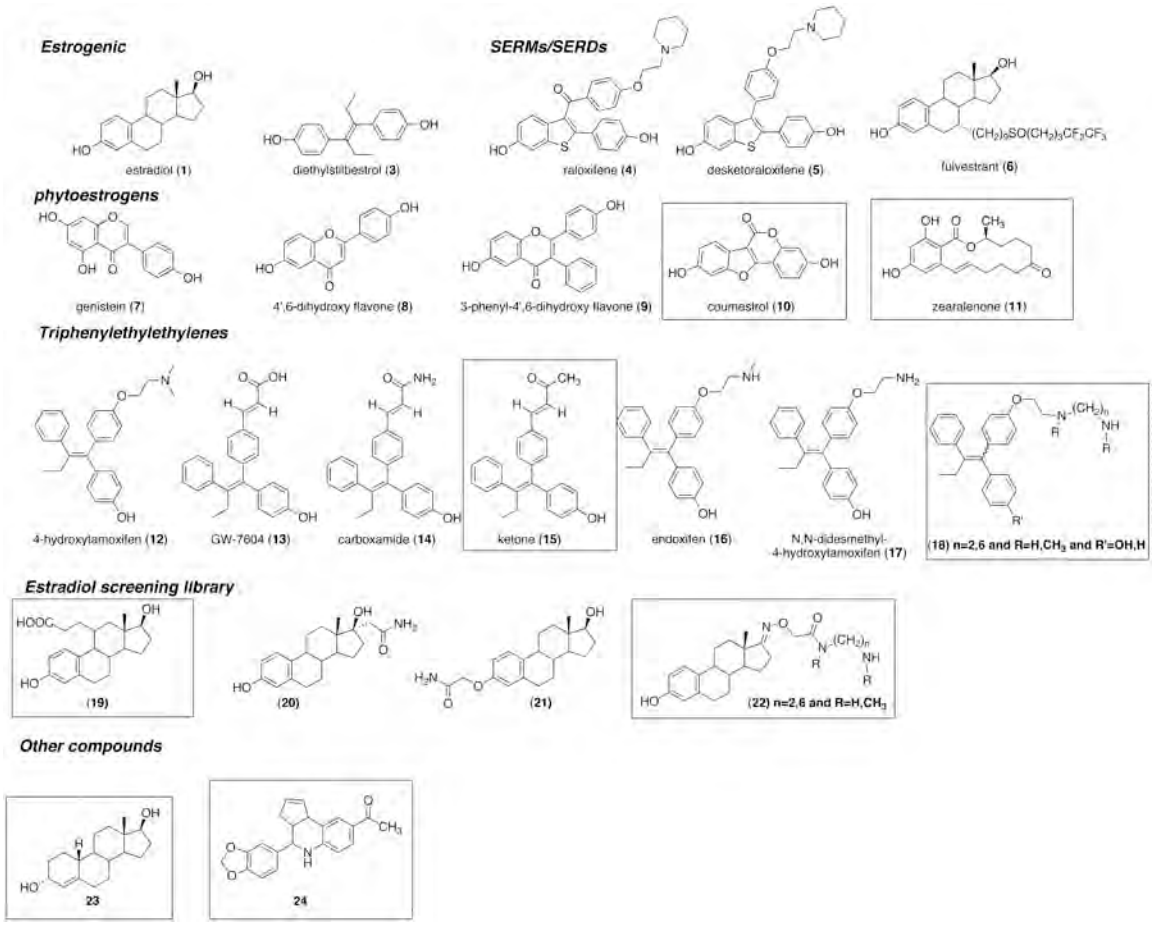


Figure 1. Initial screening panel. Compounds that were synthesized that were not part of the proposed panel are boxed in solid boxes.

In generating this panel of compounds, a number of new synthetic approaches were developed. Below is a description of these new discoveries.

<u>Synthesis of triphenylethylethylenes</u>
In order to generate new side chain analogs of 4-hydroxytamoxifen, a new synthesis was developed that greatly simplified the approach compared to previous syntheses. The first approach was developed using a monoalkylation followed by McMurray coupling to generate analogs with different side chain moieties to make compounds **16**,**17** and **18** (R=H, R'=OH, n=2). Another

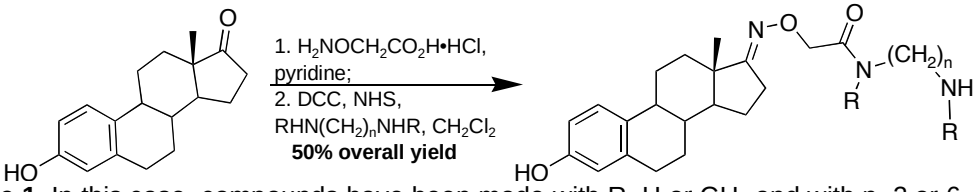
synthesis was developed to overcome inadequacies of the original plan by modifying a previously reported synthesis of 4-hydroxytamoxifen [1]. This procedure allows for the facile generation of gram quantities of 4-hydroxytamoxifen analogs and has been used to make a number of different analogs of compound **18** [2]. See attached manuscript (Trebley et al., 2006) for details.

New synthesis of GW-7604 analogs

Another set of compounds in the library based on the triphenylethylethylene scaffold is the GW-7604 series (**13**,**14** and **15**). These compounds have been synthesized using a previously reported procedure, but an improved synthesis was needed [3]. These compounds are interesting due to recent reports that their ER-modulating properties are more like pure antiestrogens like fulvestrant than SERMs like tamoxifen [4]. A new synthesis was designed and executed. This synthesis also allowed for more facile introduction of different side chains for this class of compounds. See attached manuscript currently in press (Fan et al.) for more details.

Estradiol screening panel synthesis

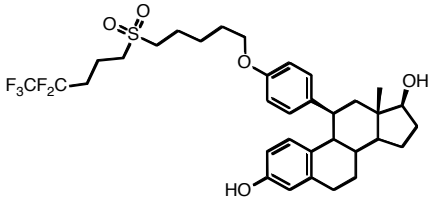
The initial plan was to attach acetamide groups to 5 different positions on the estradiol steroid scaffold- 3, 6, 7, 11, and 17. Synthesis of the 3-substituted analog was straightforward after modifying a previously reported procedure [5], but as will be described later, generated a compound that was unable to bind to estrogen receptor alpha. As a result, that substitution point is not being pursued at the current time.



Scheme 1. In this case, compounds have been made with R=H or CH_3 and with n=2 or 6.

Substitution at 17 has been accomplished through two different routes. The first involved Grignard alkylation of estrone to generate a 17-α alkyl group. This compound only has moderate affinity for the receptor. As a result, another 17-substituted compound was made by forming the oxime at the 17-position starting from estrone (**Scheme 1**). Modifying a previously reported procedure [6], a number of analogs have been synthesized containing this substitution and they have been found to possess moderate affinity for the estrogen receptor.

Difficulties were encountered with synthesizing analogs derivatized at the 6, 7 or 11 position. The decision was made to focus on substitution at the 11 position because these analogs look most like fulvestrant (also known as ICI 182,780) and RU 58,668 (**25**), antagonists in



RU 58,668 **(25)**

many rapid response assays. A new synthetic route based on the most recent literature report has been started and there is hope that the problems can be solved [7]. Once this 11-substituted analog is complete, the screening panel will be entirely finished.

New compounds

Since the submission of this proposal, there have been reports of compounds with no reported activity in regulating estrogen receptor-mediated transcription, but still possessing the ability to stimulate rapid signaling. The first molecule in this class was the estren derivative, 4-estren-3α,17β-diol (**23**), which was shown to selectively activate rapid signaling in bone without much transcriptional modulating activity [8], but the selectivity and receptor specificity has been challenged by a number of papers. [9-12] Another compound, known as G-1 (**24**), has been shown to selectively activate GPR30, an orphan GPCR that has been shown to be activated by estrogens and might be responsible for some nongenomic effects [13-15]. These compounds were purchased and used in later experiments.

Finally, a number of phytoestrogens have also been proposed to possibly modulate breast tumor proliferation [16]. A number of these compounds based on flavinoid structures were already included in the screening panel, two more non-flavinoid compounds were added to the panel, coumestrol (**10**) and the mycotoxin zearalenone (**11**). These compounds were purchased and used in later experiments.

Task 1b Test the ability of the compounds to modulate nuclear-initiated signaling by performing reporter gene assays at classic ERE promoters or nonclassical AP-1 promoters.

The overall goal of this proposal is to develop chemical tools to study rapid responses to steroid hormones. Key to accomplishing this goal is being able to correlate the ability of the compounds to bind to the nuclear receptor in vitro with the ability to directly activate the kinases and regulate the gene transcription by different transcription factors in cells. Therefore, assays for all three activities need to be developed and will be described below.

Nuclear receptor binding

There have been many assays reported to measure the binding of compounds to either the estrogen receptor. Most involve competition experiments using purified receptor or crude cell extracts and radiolabeled steroid hormone. We used a commercially available assay kit based on fluorescence polarization with purified recombinant estrogen receptor alpha and beta and a fluorescent hormone analog. The assays were performed in 96 well plates and are fairly routine. A standard competition curve for estradiol is shown in **Figure 2**. **Table 1** lists the binding affinities of any compound that has not been reported previously in the literature. From the data, it is clear that the original plans for sites were conjugation were not going to result in compounds with enough affinity, so new

conjugates were synthesized quickly and high affinity compounds were produced. It also appears that the length of the linker arm extending away from the compound is not crucial in obtaining high affinity compounds. Ultimately, the fluorescence polarization assay proved to be too inconsistent to justify the cost, so we switched to a standard radiolabeled ligand competition assay that is much more reliable and will be used for conjugate studies described later.

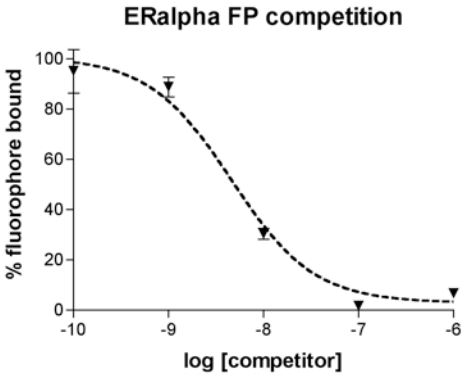


Figure 2. Estrogen receptor alpha competition binding experiment vs. 2 nM Fluormone™ with estradiol as the competing ligand. Each point represents three separate samples

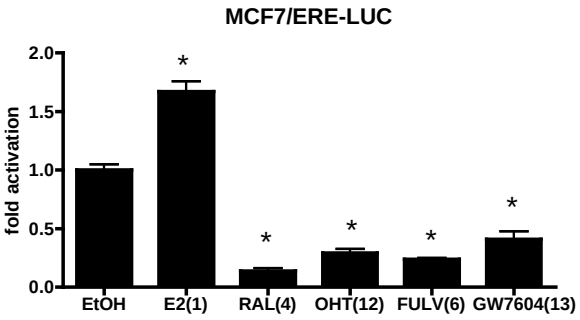


Figure 3. Luciferase reporter gene assay using vitellogenin-ERE promoter in transiently transfected MCF-7 cells. The number next to each compound refers to the structures in Figure 1.

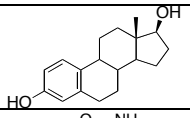
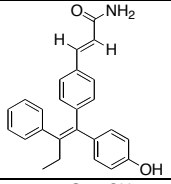
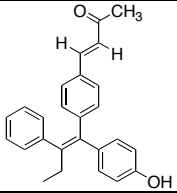
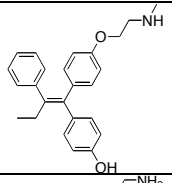
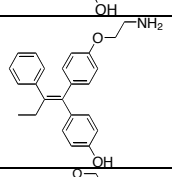
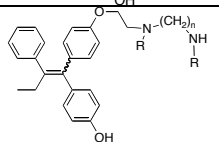
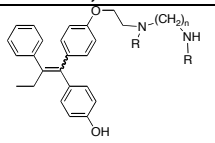
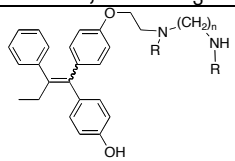
Luciferase reporter gene assays

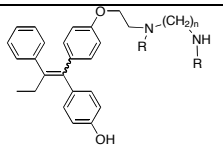
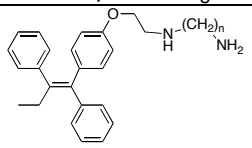
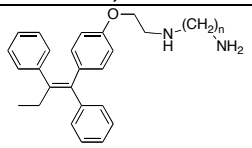
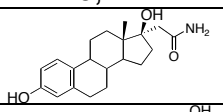
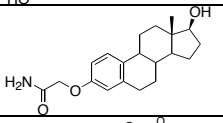
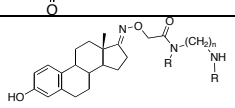
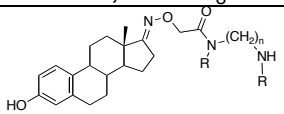
A key component of this project is measuring the estrogen receptor-mediated transcriptional activity of the compounds. For estrogen receptor, MCF-7 cells, which contain both ERα and ERβ, were transiently transfected with a luciferase reporter plasmid controlled by a simple estrogen response element (ERE)-containing promoter from the upstream region of the vitellogenin gene. The ER-negative HeLa cell line was also used for these experiments, but an expression plasmid for either ERα or ERβ was cotransfected with the reporter plasmid. A dual luciferase reporter gene system was used to normalize for transfection efficiency, meaning that an enzymatically orthogonal form of luciferase from a different species was cotransfected on a constitutively active expression plasmid. The DNA was transfected into the cells using Lipofectamine 2000 and standard protocols. After transfection, the cells were treated with drug for 1-2 days and the activities of the two luciferases were measured independently using a commercial kit. This assay is quite robust and reliable.

Figure 3 and **Table 1** show the fold activation of transcriptional activation at the ERE response element of a number of the compounds from the screening panel. As is to be expected, most of the SERMs and antiestrogens act as antagonists and estradiol and genistein act as agonists. The only real surprise was the activity of estren. This compound was reported to have no activity with estrogen receptor, but it is clear that there is some agonist activity and other papers have reported this activity as well [9,12]. The reason behind this activation is still being

explored. The activity of antagonists can be also be measured by performing a competition experiment with 10 nM estradiol. **Table 1** lists the inhibitory potencies of any compound that has not been reported previously in the literature. From the data, the potency of the compounds at repressing ER-mediated transcription correlates with binding affinity.

Table 1.

compound		Ki (nM)	IC50 (nM)
estradiol **(1)**		6.3 ± 0.2	N.D.
14		35 ± 17	110 ± 20
15		25 ± 12	55 ± 10
16		8.5 ± 3.9	40 ± 10
17		48 ±5	800 ± 400
18	 n=2, R=H	32 ± 10	150 ± 50
18	 n=2, R=CH_3	3.4 ± 2.1	39 ± 12
18	 n=6, R=H	9.8 ± 6.2	85 ± 55

18	 n=6, R=CH_3	6.2 ± 4.6	126 ± 33
18	 n=2, R=H	Yet to be det'd	150 ± 24
18	 n=6, R=H	Yet to be det'd	110 ± 32
20		850 ± 75	3275 ± 200
21		1100 ± 100	> 10 μM
22	 n=2, R=CH_3	9 ± 4	13 ± 6 (weak agonist)
22	 n=6, R=CH_3	22 ± 8	32 ± 11

The case of endoxifen

N-desmethyl-4-hydroxytamoxifen (compound **16**) was synthesized as part of the series of 4-hydroxytamoxifen analogs designed to test the feasibility of conjugating 4-hydroxytamoxifen to a polymer scaffold. During the course of the synthesis, we began collaborating with David Flockhart and colleagues at Indiana University School of Medicine. They had preliminary data that this compound, which they named endoxifen, was a metabolite of tamoxifen in women undergoing tamoxifen therapy, but was missing in women with deficient cytochrome P450 2D6 activity. We gave them some of our chemically synthesized material and they confirmed the metabolite structure with our material [17]. Together, our labs tested the activity of endoxifen in binding assays, luciferase assays, and rapid response assays. Thus far, endoxifen appears to be identical in activity and potency to 4-hydroxytamoxifen. See reference 17 for more information Further studies by Flockhart and colleagues have shown that endoxifen is at much higher concentrations in normal patients than 4-hydroxytamoxifen and should be considered a major bioactive metabolite of tamoxifen. Furthermore, patients with deficient CYP2D6 activity, either through

mutation of the gene or through the use of CYP2D6 inhibitors (such as the antidepressants fluoxetine and paroxitene) may be more likely to have a poor response to tamoxifen [18]. This work has resulted in a letter sent to the FDA warning of a potential drug interaction between tamoxifen and drugs that are known to inhibit CYP2D6..

Task 1c. Test the ability of the compounds to mimic estrogen's ability to inhibit apoptosis in breast cancer by treating a breast cell line with the compounds in the presence of taxol and testing for both early and late apoptosis events.

One of the key aspects of this project is determining the effect of various compounds on the tolerance to apoptosis that estradiol confers to ER positive breast cancer cells. We started these assays early in this project but have had some difficulty in obtaining reproducible results. Early efforts focused on using a previously reported assay for caspase 9 activity to indicate early events in apoptosis [19]. This assay has not been successful in our laboratories. We have also performed fluorescence microscopy studies to look at annexin V binding to the cell surface- a marker for the late stages of apoptosis. While some data were generated with this approach, a flow cytometry based approach would be much more statistically significant. A student is trained in the to execute these experiments, but has been focused on testing the conjugates generated in task 3. The plan is to go back to this panel once we have a firm understanding how the conjugates are affecting apoptosis and try to dissect the structure-activity relationships of this effect, particularly using analogs **18** and **22**.

Task 1d. Test the ability of the compounds to mimic estrogen's ability to rapidly initiate kinase signaling cascades known to be important in cell proliferation by treating different cell lines with the compounds and testing for modulation of kinase pathways starting with MAP kinase.

While there are many different assays that can be run to measure direct activation of the three kinases, the goal of this proposal was to start with assays that are well established. For our initial studies, we decided to focus on the direct activation of ERK1/2 in two cell lines– the ER-positive breast cancer cell line MCF-7 and the ER-negative cell line MDA-MB-231 with or without transfected ERα. For measuring direct activation of ERK1/2, the cells were serum-starved for 3 days to quiet any background MAPK signaling. Cells were then treated with drug for various time points, then the cells were lysed and the cell lysates were analyzed for total ERK and phosphorylated ERK (pERK) using previously reported Western blotting procedures [20]. While some stimulation with different compounds was seen in transfected MDA-MB-231 cells, it was clear from later experiments with fluorescent proteins that the transfection efficiency of this model system is relatively poor (less that 30%) and that most of the MDA-MB-231 cells were untransfected. As a result, experiments with transfected MDA-MB-231 cells were abandoned. In contrast, in ER-positive MCF-7 cells, estradiol stimulated ERK phosphorylation about as strongly as epidermal growth factor (EGF), which is consistent with previous reports. [21] Care is taken to not exceed an ethanol or

DMSO concentration in the media over 0.01% since higher levels of either solvent can stimulate ERK phosphorylation. The specificity of the MAPK pathway for ERK phosphorylation is shown by the inhibition of estradiol stimulation by the MEK inhibitor PD98059. Specificity for an estrogen response is shown with the inhibition of estradiol stimulation using the antiestrogen fulvestrant. The time course of activation was also determined in MCF-7 cells and is shown in **Figure 4**. The ERK activation after dosing with estradiol was maximal at 5-10 minutes with most of the activation returning back to baseline after 15 minutes.

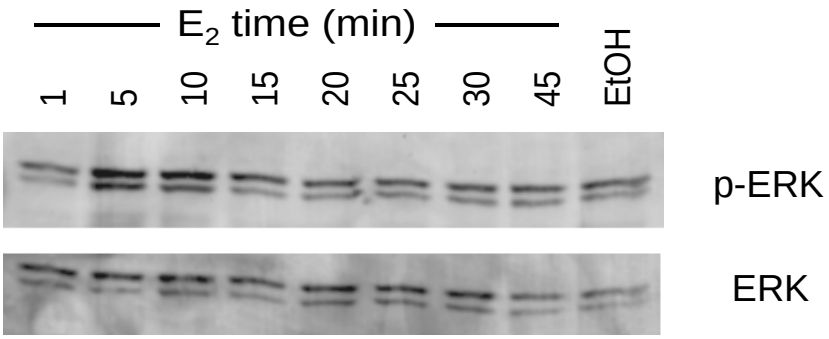


Figure 4. ERK phosphorylation in MCF-7 cells after doing with 10 nM estradiol.

The effects on ERK activation of a number of other compounds in the screening library are shown in **Figure 5**. Tamoxifen, 4-hydroxytamoxifen, estren, raloxifene and desketoraloxifene all elicited ERK phosphorylation after 15 minutes in MCF-7 cells. This activation was MAPK specific as it was inhibited by PD98059. All of the responses were estrogen receptor specific in that they activation could be blocked by fulvestrant (also known as ICI 182,780) except for the tamoxifen compounds. It appears that ERK activity *increases* in the presence of fulvestrant. This experiment has been repeated and the same result is obtained. Work is currently underway to try to understand the origin of this effect, although a recently published paper with a similar finding about OHT vs. fulvestrant modulation of ERK suggests that OHT can activate ERK through a fulvestrant-insensitive pathway [22].

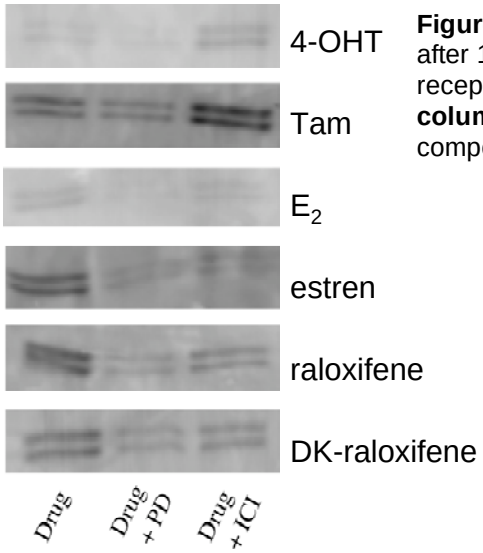


Figure 5. phospho-ERK levels in MCF-7 cells after 15 minute exposure to different estrogen receptor ligands. **column 1**-100 nM compound; **column 2**- compound + PD 98059; **column 3**- compound + fulvestrant (ICI 182,780)

Problems with activation assays

The major obstacle that faced this part of the project currently was the lack of consistent and vigorous activation of the MAPK pathway. The fold activation was usually 2-3 fold over baseline, but many times the baseline seemed to be much higher than normal and no ER-induced activation is seen. Various types of serum starved and serum-free conditions were tried as well as cell lines expressing high levels of Her2/neu. We have tried other antibodies as well as immunoprecipitating ERK and performing kinase enzymatic assays. The same issue arose looking at the phosphorylation of Akt, reported to be another downstream effector of nongenomic estrogen signaling. Ultimately, we did not find a technique for either signaling pathways that gave highly reproducible results in our hands.

There were a number of other possible solutions in the literature that we pursued. One involved making a form of the estrogen receptor that localizes to the membrane. This receptor lacked the nuclear localization site and included additional myristoylation and prenylation sites and was reported to have strong ERK activation properties [23]. In order to confirm that the receptor localized to the membrane, we first ran a luciferase reporter gene experiment with the reporter gene coupled to a classic estrogen response element-controlled promoter. In reported work by others, this receptor did not regulate transcription at an ERE promoter [23]. In our work, estradiol was still able to activate transcription from the ERE promoter, suggesting that there was perhaps still some nuclear activity. We then constructed a version of the membrane-localized receptor fused to green fluorescent protein (GFP) and transfected cells with this expression plasmid. While fluorescence was observed at the membrane, significant fluorescence was also observed in the nucleus both before and after addition of estradiol, suggesting that the targeting strategy was unsuccessful. As a result, this approach was abandoned.

Serum Response Factor Modulation

We also tried luciferase reporter gene assays using a number of different downstream transcription factors that were reported to be sensitive to changes in MAPK or PI3K activation. We focused our attention on the transcription factors that regulate transcription at the serum response element (SRE), Elk-1 and the serum response factor (SRF). Genes under control of SRE containing promoters have been reported to be expressed very quickly after estradiol treatment and do not appear to involve direct estrogen receptor modulation of the promoter. Rather, Elk-1 and SRF are modulated by estrogen receptor through the MAPK and PI3K pathways (**Figure 6**) [24,25]. In order to determine whether our screening panel had unique modulatory properties through these pathways, an SRE luciferase reporter plasmid, an SRF reporter plasmid and Elk-1 reporter plasmids (the Elk-1 reporter system consists of two plasmids) were transfected into ER-positive MCF7 cells, and the ER negative cell lines MDA-MB-231 and SKBR3. For the ER-negative cell lines, expression plasmids for either ERα or ERβ were cotransfected.

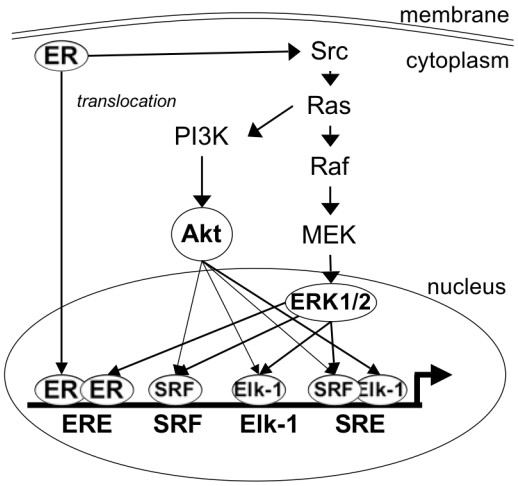


Figure 6. Proposed regulation of the transcription factors SRF and Elk-1 by crosstalk of estrogen receptor with the MAPK and PI3K pathways. SRF and Elk-1 work together at the serum response element (SRE) when they are expressed in the same cell.

The Elk-1 reporter plasmids did not show significant ligand-dependent modulation in any of the cell lines tested. In contrast, the SRF-controlled reporter plasmids did show significant ligand responses in an ER-dependent manner that was also dependent on cell context and this activity was also seen with the SRE reporter, although the relative SRF reporter response compared to vehicle was greater than the relative SRE response compared to vehicle. Using a dual luciferase reporter assay to normalize for transfection efficiency, estradiol was found to increase SRF-mediated transcription (**Figure 7A**), consistent with a previous report that indicated that SRF could be modulated by estrogen receptor through crosstalk with MAPK and PI3K [25]. Testing the other compounds in the panel, however, revealed that the selective estrogen receptor modulator (SERM) raloxifene repressed the basal activity of SRF. Somewhat surprisingly, other compounds such as the SERM 4-hydroxytamoxifen and the selective estrogen receptor downregulators (SERDs) fulvestrant and GW-7604 did not have significant effect on basal SRF activity.

A recent study has suggested that the orphan G-protein coupled receptor GPR30 regulates the transcription of *c-fos*, possibly by modifying the activity of SRF and Elk1 at the SRE contained in the c-fos promoter region [26]. In order to test the possible involvement of GPR30 in mediating the effects of estradiol and raloxifene on SRF activity, the reporter plasmids were tested in the ER-negative, GPR30-positive SKBR3 and MDA-MB-231 breast cell lines. To confirm that no endogenous ER activity was present in the either cell line, an ERE-containing reporter plasmid was first transfected into the cells and the cells were treated with either estradiol or raloxifene. No significant ligand-dependent response was seen in either cell line with either the ERE or SRF-controlled reporter unless an expression plasmid for ERα was cotransfected suggesting GRP30 does not regulate SRF activity in these cells. When the cells were cotransfected with an ERα expression plasmid, however, a strong ligand dependent response was seen with MD-MB-231, which had a similar drug response as MCF-7 cells.

Surprisingly, transfected SKBR3 cells showed a reversed profile from that seen in MCF-7 cells (**Figure 7B**). Raloxifene strongly stimulated SRF activity in SKBR3 cells transfected with ERα and estradiol repressed SRF activity. This suggests a new, cell context dependent pathway by which compounds that normally repress transcription at ERE promoters can activate transcription at other promoters.

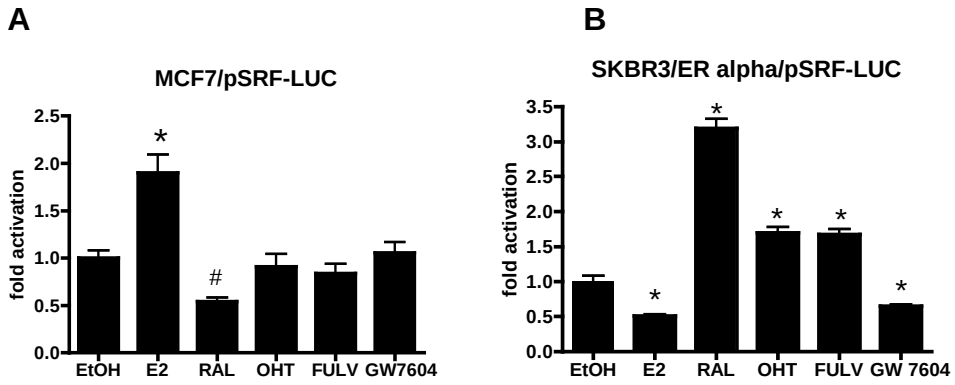


Figure 7. Effects of some of the screening panel on MCF-7 (**A**) and ERα-transfected SKBR3 cells (**B**) transfected with a luciferase reporter plasmid containing an SRF binding motif. The cells were treated with drug for 24 hours in charcoal-stripped media. The results were obtained using a dual luciferase kit to normalize for cell number and transfection efficiency and the results are displayed as fold activation over the ethanol vehicle. All drugs were tested at 1 μM concentration. Activity is reported as fold activation compared to the ethanol vehicle. * represents responses differing from the vehicle response with $p < 0.01$. # represents responses deviating from the vehicle response with $p < 0.05$.

Increasing concentrations of ERα expression vector increased the overall level of SRF-mediated activity, but the same relative level of stimulation by raloxifene compared to the vehicle control was observed, so the inversion of the raloxifene response in ERα-transfected SKBR3 cells compared to MCF-7 cells is not due to major differences in receptor expression. A normal dose response profile with raloxifene in these transfected SKBR3 cells was observed, suggesting that the inversion of the raloxifene response in the SKBR3 cell line is not simply a non-specific response to high concentrations of raloxifene. Inhibitors of different kinase signaling pathways were used to determine whether PI3K or MAPK pathways played a role in the effects of ERα on SRF activity. At the ERE-containing promoter, some reduction in the overall level of activation was seen for all drugs in the presence of either or both inhibitors, but the relative levels of activation for estradiol and raloxifene compared to vehicle was unchanged. In contrast, the overall transcriptional activity at the SRF reporter plasmid did not change significantly with inhibitor treatment, but the extent of raloxifene activation of SRF activity decreased approximately 30-40% in the presence of either the PI3K inhibitor or the MAPK inhibitor. This strongly suggests that both the MAPK and PI3K pathways play a role in the stimulation of SRF activity by raloxifene and ERα. Unfortunately, addition of both inhibitors simultaneous was toxic to the cells, so the redundancy of the signaling pathways could not be explored. Interestingly, the repression of SRF activity by estradiol in ERα-transfected

SKBR3 cells did not appear to be affected by either or both inhibitors, suggesting a different mechanism of action for estradiol repression compared to raloxifene activation. A manuscript detailing all of these results is currently in revision.

There are still obviously a number of issues we must explore before claiming SRF reporter plasmid activity as a valid downstream assay for rapid estrogen signaling. The most important experiment is to correlate compound's ability to modulate ERK and Akt phosphorylation with its SRF profile. In MCF-7 cells, no correlation has been found between a compound's ability to stimulate ERK phosphorylation and its SRF activity, but previous reports suggest that SRF is regulated by PI3K in MCF-7 cells and not by MAPK [24,25]. The key experiments will be the Akt phosphorylation assays, which are underway. The major obstacle to determining whether SRF response in SKBR3 cells is downstream of rapid signaling is poor transfection efficiency of the estrogen receptor. If transfection efficiency is low, large number of cells that are not responding at all will dilute the overall extent of ERK and Akt phosphorylation assays in cells successfully transfected with the estrogen receptor. To get a better response, we searched for an ER positive breast cancer cell line that showed the same profile as SKBR3 cells and were unsuccessful, increasing our concern that the result observed were simply an artifact of losing ER expression. As this project continues, we are making an SKBR3 cell line stably transfected with an ER alpha expression vector. This will allow us to determine the correlation between SRF activity and MAPK and PI3K activation and also determine whether there are correlations between the SRF response to different compounds and cell properties such as proliferation, resistance to apoptosis and antiestrogen resistance. Those transfections are currently underway.

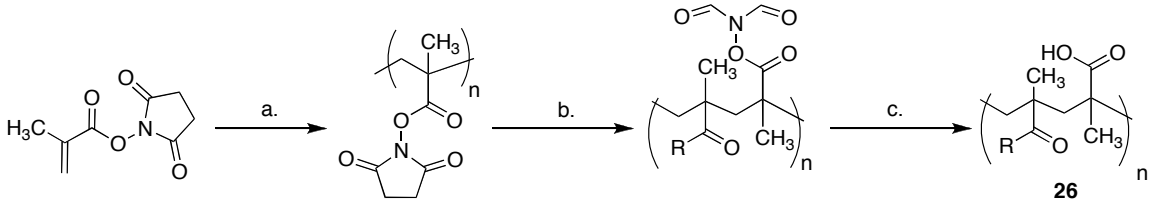
Task 2. Test the potential role of estrogen receptor alpha (ER alpha) or ER beta in rapid signaling, by performing assays with various N-terminal deletion and chimera mutants of ER alpha and ER beta.
All of the necessary mutants are prepared and active in luciferase reporter gene assays. The task has been curtailed by lack of a rapid signaling assay is in place. We do know that there are some differences in the SRF modulation by ER beta compared to ER alpha. The main difference in ER beta transfected SKBR3 cells was that the activation at the SRF-modulated promoter was higher with tamoxifen than with raloxifene, the opposite of what was seen with ER alpha transfected cells. Differences between tamoxifen and raloxifene responses and ER alpha and ER beta have been seen previously [27,28].

Task 3. Test the role of cell surface receptors in rapid estrogen signaling, by developing cell-impermeable, non-proteinaceous estradiol conjugates.

Task 3a. Determine the feasibility of using polymer-conjugated estrogen ligands as probes of ER function by conjugating active estradiol & tamoxifen analogs to polymers synthesized using atom transfer radical polymerization (ATRP) and testing for their ability to bind to ER in vitro.

Synthesis and ER binding

In designing the tamoxifen-polymer probes, poly(methacrylic acid) was chosen as the scaffold due to its ease in coupling reactions and its ability to be synthesized with a narrow molecular weight range using controlled radical polymerization [29]. A polymer scaffold with a molecular weight of approximately 12,500 and a polydispersity index of 1.07 was synthesized with N-hydroxysuccinimide activated ester side chains well suited for facile attachment of ER ligands. The activated ester-containing polymer was conjugated to 4-hydroxytamoxifen analog **18** (n=6, R=H) and the remaining unconjugated side chains were hydrolyzed to carboxylic acids to give the conjugate (**Scheme 2**). Since the analog is a potent antiestrogen on its own, samples were dialyzed exhaustively in water to ensure that all free ligand was removed. The resulting conjugate showed that approximately 40% of the side chains were conjugated with ligand.



Scheme 2. Reagents and conditions: (a) 2-bromo-2-methyl-(2-hydroxyethyl) propanoate, CuBr, 2,2'-bipyridine, DMSO, 100 ˚C. (n=150); (b) **18 (R=H,n=6)**, NEt_3, DMF, 90 ˚C, 72 hr; (c) 2M NaOH, 12 hr.

Polymer conjugate **26** was then tested for its ability to bind to ER alpha and ER beta using a radiolabeled estradiol competition assay with purified recombinant receptor. In these assays, shown in **Figure 8**, the conjugate was able to bind to both estrogen nuclear receptors. The IC50 values were 35 ± 30 nM for ER alpha and 27 ± 20 nM for ER beta. In the same assay, the unconjugated ligand had IC50 values of 15 ± 5 nM for ER alpha and 9 ± 5 nM for ER beta. For comparison, estradiol was found to have IC50 values of 0.5 ± 0.1 nM for ER

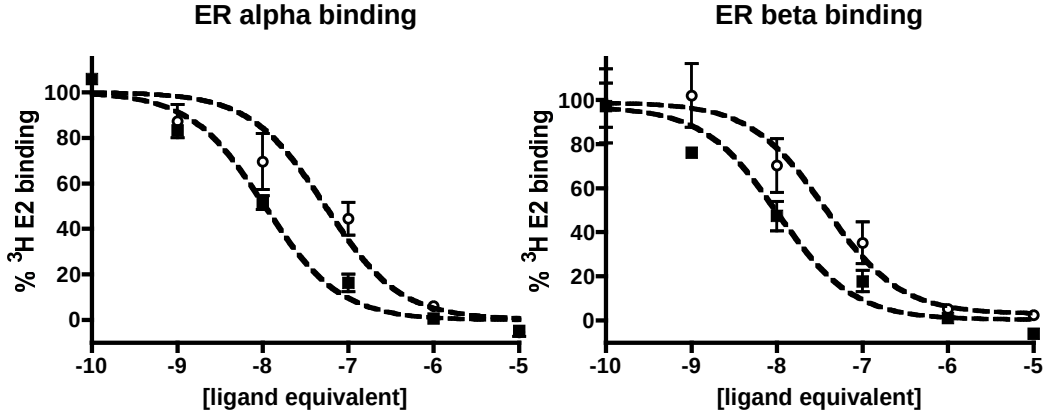


Figure 8. Binding of compound **18** (n=6, R=H) (black squares) and its polymer conjugate **26** (open circles) to ER alpha (left) and ER beta (right) as measured using a radiolabled ligand competition binding assay. The lines represent the best fit to a one binding site competition model using non-linear regression analysis.

alpha and 0.3 ± 0.3 for ER beta and 4-hydroxytamoxifen had IC50 values of 1 ± 0.3 nM for ER alpha and 8 ± 5 nM for ER beta. The numbers for estradiol and 4-hydroxytamoxifen are consistent with other number reported in the literature [30]. The conjugate shows dramatically improved affinity compared to protein-based steroid hormone conjugates. Commercially available bovine serum albumin (BSA)-estradiol conjugates have been used to target membrane estrogen receptors, but their use has been highly controversial with reports of high levels of unconjugated steroid, slow binding to the estrogen receptor, poor affinity for the receptor in vitro (IC50 levels greater than 100 nM) and unusual biological responses that are possibly due to the BSA portion of the conjugate [31,32].

Task 3b. Develop cell-impermeable polymer scaffolds suitable for cell-based assays by synthesizing well-defined polymers of different sizes and derivatizations from a single monomer unit using ATRP and testing for their general utility in biological screens.

Conjugate stability and protein binding

The polymers were tested for their chemical and enzymatic stability. To test this a conjugate was made that contained both the 4-hydroxytamoxifen analog **18** (n=6, R=H) and fluoresceinamine. Since these polymers were larger than free fluorescein, a significant difference was seen in the fluorescence polarization values for the polymer-fluorescein conjugates compared to free fluorescein. Likewise, aggregation of the polymers by serum proteins should result in a much greater increase in the fluorescent polarization of the conjugates. Hydrolysis of the fluorescein from the polymers using concentrated NaOH, followed by neutralization resulted in samples with significantly lower polarization values. No change in fluorescence polarization was noted after the addition of 10% fetal bovine serum and incubation for 2 days at 37 ˚C. This suggests that the conjugates are relatively stable in serum and that large aggregates are not being formed between serum proteins and the conjugate.

Cell-impermeability

One interesting feature of the other conjugates of estrogen receptor ligands is their reported cellular activity. The estradiol-protein conjugates have been reported to localize to the plasma membrane and an estradiol-dendrimer conjugate localized to the cytoplasm in ER-positive cells [31-34]. None of the conjugates were capable of activating estrogen-receptor mediated transcription, although their ER antagonist effects were not tested. To test the effect of the hydroxytamoxifen-polymethacrylate conjugate on ER-mediated transcription, the ER negative breast cancer cell line MDA-MB-231 was transiently transfected with an expression vector for human ER alpha or human ER beta and a reporter plasmid containing the luciferase gene controlled by a classic estrogen response element-containing promoter sequence. After transfection, the cells were treated for 18 hours with different concentrations of conjugate and then the amount of luciferase expression was measured using a luminescence assay. Compound **18**, conjugate **26** and the unconjugated polymethacrylic acid were unable to

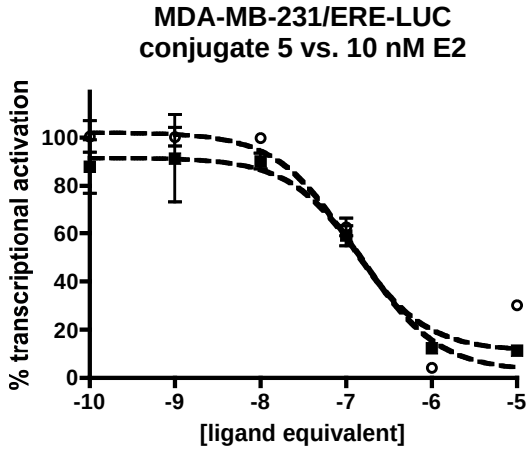


Figure 9. Competition of polymer conjugate versus 10 nM estradiol in transient transfection assays of MDA-MB-231 cells with an ERE driven luciferase reporter gene and either ER alpha (closed squares) or ER beta (open circles) The numbers are plotted on the y-axis as the percent signal compared to the activation with 10 nM estradiol alone. Curve represents the best fit to a single-site competition binding model.

activate transcription as agonists at an ERE-controlled promoter (data not shown). However, the polymer conjugate was able to act as an antagonist of estradiol-stimulated transcription (**Figure 9**). The conjugate inhibited transcriptional activity induced by 10 nM estradiol in ER alpha transfected cells with an IC50 of 220 ± 130 nM and in ER beta transfected cells with an IC50 of 70 ± 30 nM. This is roughly 10 fold worse than the unconjugated ligand alone and approximately 50 fold worse than 4-hydroxytamoxifen. No activity was seen in MDA-MB-231 cells transfected with only the reporter plasmid and no estrogen receptor expression vector, suggesting dependence on the presence of estrogen receptor. To the best of our knowledge, this is the first time that a macromolecular conjugate of a hormone has been reported to modulate a nuclear receptor's transcriptional activity. A manuscript has been submitted detailing these results.

Considering that unmodified polymethacrylic acid conjugates are usually not taken into the cell to a high degree, let alone transported to the nucleus, the antagonist activity of our hydroxytamoxifen-polymer conjugate was unexpected. The polyanionic nature of the polymer is usually masked as amides or by polycationic binding partners before the polymer can be taken efficiently into cells and then uptake-enhancing peptides are usually also included to get significant uptake [35-37]. To determine whether cells were taking up the polymers, a fluorescent hydroxytamoxifen polymer conjugate was used. As shown in **Figure 10**, the tamoxifen conjugate is present inside the cell in high amounts, confirming that this scaffold is not cell impermeable. The conjugate appears to be taken into endosomes early in the process as evidenced by the punctate pattern in the cytoplasm. Eventually, however, the fluorescence spreads to the rest of the cytoplasm and, at higher concentrations, is seen in the nucleus.

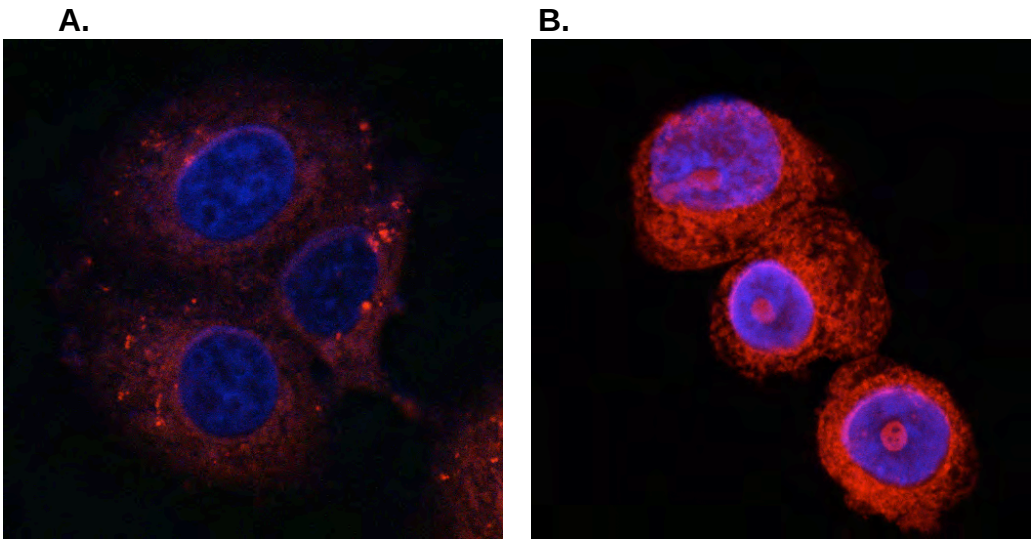


Figure 10. Merged images of MCF7 cells treated with conjugate **26** labeled with a red BODIPY-TR fluorophore obtained using confocal microscopy. Red represents label from conjugate. Blue represents DAPI nuclear stain. Panel A represents cells treated with 1 μM ligand equivalent for 12 hours. Panel B represents cells treated with 10 μM ligand equivalents for 12 hours

Other experiments showed that cells take up the fluorescent tamoxifen conjugate to a greater degree than the non-tamoxifen conjugated polymer, suggesting the addition of tamoxifen actually increases uptake. Additionally, the addition of 4-hydroxytamoxifen greatly decreased the uptake of the polymer. This suggests that something might be specifically transporting the conjugate into the cell. This would be an important (if somewhat serendipitous) discovery because tamoxifen was always considering to be taken up by cells via passive diffusion. An active transport process could potentially be studied further and manipulated to potentially increase the efficacy of tamoxifen therapy.

One alternative explanation for the transcriptional activity of the polymer conjugate is that free hydroxytamoxifen analog is present in the sample or is being released by conjugate degradation. As stated previously, the polymer conjugates are dialyzed extensively and no free small molecule appears to be present in the initial sample as measured by HPLC. HPLC showed that conjugated ligand is present initially in at least 10^5 fold excess compared to unconjugated ligand, therefore it is unlikely that the biological activity of the polymers is due to unconjugated ligand present from the beginning of the experiment. It does not, however, rule out possible degradation of the conjugate by cells and release of the drug. Similar inhibitory profiles are seen with the conjugate under both serum-rich and serum free conditions, suggesting that there are no serum components causing conjugate degradation. We have not yet been able to completely rule out the possibility that the 4-hydroxytamoxifen analog is released after cleavage by some sort of membrane-associated hydrolase. While amide bonds can be hydrolyzed, amide bonds are generally not

considered to be a linkage of choice for biodegradable linkers, especially considering the relatively short time frame of the experiments [38]. In addition, amide bonds have been used with other steroid conjugates that have not modulated transcriptional activity or shown any form of degradation [39-41]. As a result, we believe that the conjugate is taken into cells and then the receptor either binds to free ligand released intracellularly through lysosomal degradation or that the conjugate somehow can enter cells and bind to the receptor intact. In either case, this conjugate represents a new paradigm in delivering antiestrogens to tumor cells

The task still remains for us to generate a truly cell-impermeable conjugate. To that end, we began to synthesize conjugates that were either much larger or much more polar. The rationale behind larger conjugates was that larger conjugates might now be transported into the cell as efficiently as the polymer conjugates, which were more like the size of a protein. We first used polymer-coated fluorescent nanocrystals with activated esters on the surface of the nanocrystal. These nanocrystals were a new type of experimental nanocrystal made by a collaborator. The 4-hydroxytamoxifen conjugate with a six carbon linker was then attached via standard amide coupling procedures and the conjugated nanocrystals were washed and characterized for binding and cellular activity (**Figure 11**). A number of different ratios of ligand per nanocrystal were made, ranging from 20 ligands per particle to 200.

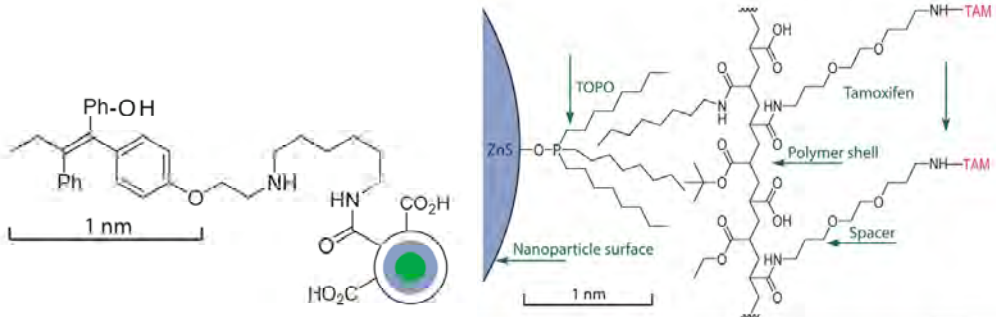


Figure 11. 4-hydroxytamoxifen conjugated polymer coated nanocrystals. Figure on the left (not drawn to scale) shows linkage of the ligand. Figure on right represents nature of polymer coating and nanocrystal. Overall, the particle is believed to be roughly 30 nM across.

The nanocrystal conjugates exhibited cellular uptake that appeared to be localized to the cytoplasm, but the no effect on transcriptional activity was seen. While we were optimistic that this suggested that the conjugated nanocrystals were bioactive and localized to the cytoplasm, binding experiments with purified ER in vitro showed no binding of receptor to the conjugates. This suggests that the ligands are likely not accessible to receptor and will not be active in the cellular assays. We believe the lack of activity is either due to the nature of the polymeric coating, which relies on noncovalent hydrophobic interactions to conjugate ligands to the nanocrystals or the length of the linker between the crystal and the drug. The hydrophobic ligand may be either pointing toward the inside of the polymer or is too close to the particle to bind. To solve this issue, we

have begun to use commercially available particles using much more rigid crosslinked polyethylene glycol (PEG) coatings and longer linkers between particle and drug. This should address whether the nanoparticle can localize to either the cytoplasm or the plasma membrane and elicit nongenomic estrogen signaling.

Even with the inactive nanocrystals, cellular uptake still occurred. This runs counter to the original goal of the research, which was to probe signaling events that originated on the plasma membrane. During the course of the nanocrystal conjugate experiments, one batch of conjugates showed no cellular uptake but was still present on the plasma membrane. These nanocrystals differed from the other batches because they were significantly larger, which seemed to happen when one polymer wrapped around more than one nanocrystal. Like their smaller counterparts, the larger conjugates did not bind to ER in vitro in binding assays, but it does suggest that if one could make a bioactive particle, it could remain on the plasma membrane if it were big enough. Current studies are focused on synthesizing these types of conjugates.

Task 3c. After establishing the ideal polymer scaffold, active compounds will be coupled to the polymers and tested for their ability to elicit rapid steroid hormone responses in the different assays. (Months 24-36)

The tamoxifen polymer conjugate was active in the ERK activation assay, but the finding that the conjugates were not cell-impermeable has put further study on this question on hold until a better, more cell-impermeable conjugate is found (**Figure 12**). Besides the particle-based conjugates described above, another approach that has been taken is to conjugate the tamoxifen analog to the highly polar Alexa-Fluor 486 Dye. This dye was conjugated to estradiol in order to localize GPR30 and was only able to bind to receptors inside the cell after cell permeabilization [14]. Synthesis of this compound is currently underway.

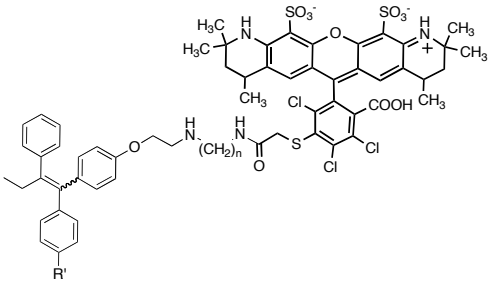


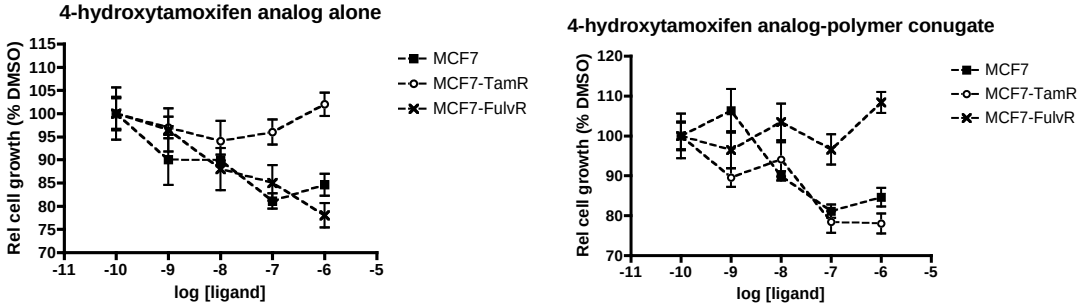
Figure 12. Alexa 486-OHT conjugate

Task 4. Test the potential role of rapid estrogen signaling in breast cancer proliferation and survival, by treating various breast cell lines with selective compounds discovered above and measuring changes in cell growth, cytotoxicity and apoptosis

Overall, we have not had much luck in the development of selective compounds. Compounds reported to have selective nongenomic activity (estren) were not selective and our cell-impermeable conjugates were not cell-impermeable. The polymer conjugates still represented new compounds and were tested for their effects on breast cancer cell proliferation in the lab of our collaborator Ken Nephew. The Nephew lab has tested the polymer-4-hydroxytamoxifen analog conjugates against resistant breast cell lines and has discovered that the

polymer conjugated to the 4-hydroxytamoxifen analog is effective at inhibiting proliferation of both tamoxifen-sensitive and tamoxifen-resistant cells (Figure 13). Even though the analog alone is not effective against these resistant cells, conjugating the drug to the polymer overcomes the resistance mechanism. The polymer conjugate is not effective against fulvestrant resistant cells, suggesting that the polymer attachment converts a SERM like 4-hydroxytamoxifen into a compound with a mechanism of action like fulvestrant. A key feature of fulvestrant's mechanism of action is the degradation of ER. When this assay was performed with the polymer conjugate, no ER degradation was seen.

Figure 13. *left* effect of 4-hydroxytamoxifen analog (compound **18** (R=H, n=6) on proliferation of different strains of MCF7 cells including strains grown in the presence of tamoxifen (TamR) or fulvestrant (FulvR). *Right.* effect of 4-hydroxytamoxifen analog conjugated polymer **26** on proliferation of the same strains of MCF7 cells. The resistant cells are ER positive, but the mechanisms of resistance are still under study.



These exciting results are being followed up with further studies into the overall mechanism of action, but it is clear that the conjugates are working through a novel mechanism of action. The additional fact that polymeric drugs can be directed toward or away from specific tissues (such as the brain, origin of hot flashes), means that these polymer conjugates could be developed into new experimental therapeutics with possible activity against ER positive, tamoxifen resistant breast cancer with improved side effect profile. We are currently working on optimizing the properties of these polymer conjugates in preclinical models with the goal of trying to translate these molecules into experimental therapeutics.

Key Research Accomplishments

- New synthesis of 4-hydroxytamoxifen analogs results in best method reported to date to make these series of important compounds.
- New synthesis of GW-7604 and analogs that is much higher yielding than previously reported synthesis
- New 4-hydroxytamoxifen analogs with high affinity for estrogen receptor and potency in cell-based assays
- First reported synthesis and testing of endoxifen, a major, bioactive metabolite of tamoxifen that may be an important indicator of tamoxifen response in breast cancer patients.

- First report of raloxifene and desketoraloxifene acting as agonists of rapid, estrogen-induced ERK phosphorylation.
- First use of ATRP to generate polymer conjugates capable of binding to the estrogen receptor.
- First report of macromolecular tamoxifen conjugates capable of stimulating rapid ERK phosphorylation.
- First reported example of specific uptake of tamoxifen polymer conjugates by breast cancer cells.
- First example of conjugate targeting nuclear receptors that behaves as highly potent transcriptional antagonists.
- New synthesis of GW-7604 and analogs that is much higher yielding than previously reported synthesis
- First reported discovery of cell-context dependent modulation of SRF transcriptional activity by estrogen receptor.
- First report of SERMs acting as strong activators of SRF signaling as well as first example of a unique response for GW-7604 compared to raloxifene and tamoxifen. .
- First synthesis of nanocrystals conjugated to steroid hormone receptor modulators.
- First report of steroid hormone conjugates that are effective against antiestrogen resistant breast cancer cell lines.

Reportable Outcomes

Manuscripts/abstracts (included in appendix)

1. Rickert, E. L.; Trebley, J. P.; Peterson, A. C.; Morrell, M. M.; Weatherman, R. V., Synthesis and characterization of bioactive tamoxifen-conjugated polymers. Biomacromolecules. In submission.

2. Trebley, J. P.; Rickert, E. L.; Diaz, N.; Behr, K. E.; Weatherman, R. V., Ligand and cell-context dependent modulation of serum response factor by estrogen receptor. Chemistry & Biology. In revision.

3. Dobrydneva, Y.; Weatherman, R. V.; Trebley, J. P.; Morrell, M. M.; Fitzgerald, M. C.; Fichandler, C. E.; Chatterjie, N.; Blackmore, P., Tamoxifen stimulates calcium entry into human platelets. Journal of Cardiovascular Pharmacology. In revision.

4. Fan, M.; Rickert, E. L.; Chen, L.; Aftab, S. A.; Nephew, K. P.; Weatherman, R. V., Characterization of molecular and structural determinants of selective estrogen receptor downregulators. Breast Cancer Research & Treatments 2007. In press

5. Weatherman, R. V., Untangling the estrogen receptor web. Nat Chem Biol 2006, 2, (4), 175-6.

6. Trebley, J. P.; Rickert, E. L.; Reyes, P. T.; Weatherman, R. V., Tamoxifen-based probes for the study of estrogen receptor-mediated transcription.

Ernst Schering Res Found Workshop 2006, (58), 75-87.

7. Dobrydneva, Y.; Weatherman, R.; Trebley, J.; Fitzgerald, M.; Blackmore, P., Tamoxifen and its analogs as calcium channel modulators in platelets: SAR study of triphenylethylene compounds. FASEB J. 2006, 20, (5), A1289-A.

8. Johnson, M. D.; Zuo, H.; Lee, K. H.; Trebley, J. P.; Rae, J. M.; Weatherman, R. V.; Desta, Z.; Flockhart, D. A.; Skaar, T. C., Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. Breast Cancer Research & Treatments 2004, 85, (2), 151-9.

Presentations

1. Speaker, Nuclear Receptor Drug Discovery Session, American Chemical Society Central Region meeting, Indianapolis, May 2004.
2. Speaker, American Association of Colleges of Pharmacy meeting, Cincinnati, July 2005.
3. Speaker, Department of Basic Medical Sciences, Indiana University-Bloomington, April 2005.
4. Speaker, Schering Foundation Workshop on Chemical Genomics, Berlin, Germany, April 2005.
5. Speaker, Breast Cancer Prevention and Control Group, Purdue University, February 2007.
6. Poster presenter, Era of Hope Breast Cancer Research Meeting, Philadelphia, June 2005.
7. Poster presenter, Gordon Research Conference on Bioorganic Chemistry, June 2004, 2007
8. Poster, National Medicinal Chemistry Symposium, Madison, WI, June 2004.
9. Poster, Purdue University Cancer Center Research Meeting, West Lafayette, August 2005, 2006, 2007.
10. Poster, Midwest Endocrinology Conference Research Meeting, Madison, WI, June 2005.
11. Poster, Midwest Area Medicinal Chemistry graduate Student Symposium, June 2005, 2006, 2007.
12. Poster, American Society of Biochemistry and Molecular Biology Research Meeting, San Francisco, March 2006.
13. Poster, Advances in Breast Cancer Research Symposium, Northwestern University Annual Basic Science Colloquium, Chicago, Illinois, May 2007.
14. Poster, ENDO 2007, Toronto, Canada, June 2007.

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People receiving support on this grant
Ross Weatherman, P.I.
Joseph Trebley, Melinda Morrelll, Priscilla Reyes, Emily Rickert, Naomi Diaz- grad students

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1. Melinda Morrell, M.S. in Medicinal Chemistry and Molecular Pharmacology, 2004
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1. Mindy Morrell, employment with Med Institute, West Lafayette, IN
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3. Joe Trebley, Ph.D., Technology Manager, Office of Technology Commercialization, Purdue University Research Foundation.
4. New collaborations with David Flockhart, Indiana University Cancer Center, Ken Nephew, Indiana University Cancer Center, Yuliya Drobydneva, Eastern Virginia College of Medicine,

Conclusions
We made significant progress in exploring the role of integrating nongenomic signaling in breast cancer prevention and treatment. We have synthesized all the planned compounds including a few more that were not in the original plan. We had difficulty finding robust and reproducible assays for the rapid response, but have discovered a number of new responses that might ultimately prove the importance of these responses. Going forward, most of the effort will be focused on finding robust assays to measure these rapid responses, exploring the possible utility of our cell-permeable conjugates and designing new cell-impermeable conjugates.

In terms of the new knowledge we have obtained thus far and its importance to breast cancer, we have been the first to synthesize a newly identified novel, bioactive metabolite of tamoxifen that may play a major role in determining the success of tamoxifen therapy. We have also shown that SERMs like tamoxifen and raloxifene can act similarly to estrogen in activating rapid responses. This agonist activity mimics the effects seen in some tissues and in tamoxifen resistant tumors; understanding the molecular determinants of this agonist

activity could help produce better treatments and chemopreventive agents for breast cancer. We also have found a new cell context dependent response to estrogens and antiestrogens. This agonist activity of antiestrogens and the antagonist effects of estrogens mimic the effects of these drugs seen in some tamoxifen resistant tumors. In addition, we also found one compound (GW-7604) that is an antagonist in both cell contexts, suggesting that this compound could potentially be used to treat some tamoxifen resistant tumors. Understanding the molecular determinants of this agonist activity could help produce better treatments and chemopreventive agents for breast cancer.

We have also synthesized the first polymeric conjugates of an estrogen receptor ligand and have shown that these conjugates are highly potent antagonists of ER action and can inhibit the proliferation of both tamoxifen-sensitive and tamoxifen-resistant breast cancer cell lines. We are excitedly pushing forward with discovering the clinical utility of these conjugates and their molecular mechanism of action. With the potential ability to target polymeric drugs to specific tissues, I am very enthusiastic that these molecules could have a significant impact on breast cancer patients in the future. Funding has been obtained to continue this project for the next five years. The Idea award was a critical component to obtaining this funding as well as enabling this junior investigator to continue his research.

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Tamoxifen-based Probes for the Study of Estrogen Receptor-Mediated Transcription

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Abstract. The nuclear receptors are ideal targets to control the expression of specific genes with small molecules. Estrogen receptor can activate or repress transcription though a number of different pathways. As part of an effort to develop reagents that selectively target specific transcriptional regulatory pathways, analogs of 4-hydroxytamoxifen were synthesized with variations in the basic side chain. In vitro binding assays and cell-based luciferase reporter gene assays confirm that all the derivatives have high affinity for the receptor and high potency at repressing direct estrogen receptor-mediated transcription.

Introduction

One of the ultimate goals of chemical genomics is to study the role of a specific protein by directly altering its activity with a small molecule. This could be performed either at the protein level by direct binding or at the transcriptional level by modulating the expression of its gene. Reagents such as small interfering RNA (siRNA) that block the production of protein have great utility, but small molecules that could either block or activate transcription of specific genes at specific time points would have a dramatic impact on discerning the role of a specific protein in cellular processes. [1] One necessary component for developing these tools is a better understanding of the molecular mechanisms of transcriptional regulation and how small molecules can affect this complex process. [2]

Nuclear receptors such as the estrogen receptor (ER) represent an ideal system in which to study the effect of small molecules on the modulation of gene expression. Most nuclear receptors are ligand-dependent modulators of transcription, thus providing a tool to study the molecular mechanisms by which gene transcription is regulated. Nuclear receptors can activate or repress transcription upon ligand binding depending on the structure of the ligand, the nature of the promoter and the cell type. [3] The estrogen receptor is a particularly interesting member of the nuclear receptor family because its effects on transcription can vary greatly depending on the ligand structure and the cellular context. For example, estradiol (**1**) has been shown to activate the expression of the *c-Myc* gene in breast cell lines and the breast cancer drug tamoxifen (**2**) antagonizes this activation (**Figure 1**). [4] In a

uterine cell line, however, tamoxifen and estradiol both activate *c-Myc* expression. Other ER ligands with very similar structures to tamoxifen antagonize *c-Myc* expression in both types of cell lines. This tissue-dependent response profile of tamoxifen has therapeutic importance because the ER-agonist effects of tamoxifen in the uterus and in tamoxifen-resistant breast tumors are major obstacles to improving the success of tamoxifen therapy. These different response profiles allow for comparison of the different transcriptional states to help elucidate the molecular mechanisms underpinning the selective modulation of specific subsets of genes.

It is well known that estrogen receptor regulates gene transcription by binding to specific DNA sequences in the promoter region, but ER can also regulate gene transcription through indirect means. Estrogen receptor can directly interact with other transcription factors such as AP-1 and alter their activity, but it can also rapidly activate signal transduction proteins such as ERK and Akt which can then activate downstream transcription factors such as Elk-1 and serum response factor (SRF). [5, 6] The activation of some of these rapid signaling occurs more prominently in cells in which tamoxifen acts as an estrogen receptor agonist, suggesting that the overall response profile of tamoxifen is tied to its ability to stimulate estrogen receptor crosstalk with other signal transduction pathways. [7] Some evidence suggests that these rapid signaling events are initiated from the plasma membrane. [8] Molecules that could selectively target only these crosstalk pathways would be very useful in delineating their role in the overall responses to tamoxifen. The work detailed here describes the synthesis and testing of tamoxifen analogs suitable for conjugation to other molecules such as fluorophores, affinity tags and cell-impermeable polymer scaffolds in order to better understand the role of crosstalk signaling in the control of estrogen receptor-mediated transcription.

Results and Discussion

Synthesis of 4-hydroxytamoxifen analogs (Scheme 1)

The key issue in making tamoxifen analogs suitable for conjugation to other moieties is the placement of the attachment point. One obvious location for attachment is the amine on the basic side chain. Based on the structure of 4-hydroxytamoxifen, the most potent form of tamoxifen, bound to the ligand binding domain of estrogen receptor alpha (ERα), the basic side chain extends out away from the interior of the binding pocket. [9] It has also previously been shown that endoxifen (**5**), a primary, bioactive metabolite of tamoxifen, can bind to the estrogen receptor both *in vitro* and in cells with only small decreases in affinity compared to 4-hydroxytamoxifen. [10] Based on this evidence, a number of analogs of 4-hydroxytamoxifen with different lengths of alkylamine side chains were synthesized.

The compounds were synthesized by using a modification of a previously reported synthesis of 4-hydroxytamoxifen. [11] The triphenylethylethylene scaffold can be synthesized as the diphenol (**3**) in a single step from commercially available start-

ing materials and then monoalkylated with dibromoethane. The resulting compound (and every compound hereafter) is generated as a mixture of *E* and *Z* isomers, but the two forms readily interconvert at room temperature. Previous work with 4-hydroxytamoxifen has shown that despite this interconversion, the Z isomer is almost exclusively bound by the receptor both *in vitro* and *in vivo*. [12]

Coupling to different amines provided the different compounds for testing. Since the optimal distance between the tamoxifen scaffold and any conjugate is not known, alkyldiamines with two and six methylene unit spacers were synthesized. Previous work has indicated that the methylation state of the amines could also be important in increasing the affinity of ligands for the estrogen receptor, so analogs with methylated amines were also synthesized.

In vitro binding assays

The binding affinity of the compounds for estrogen receptor alpha was measured using a fluorescence polarization-based competition assay using purified full-length human estrogen receptor alpha. Displacement of a fluorescent ER ligand from the receptor by the competitor results in a decrease in the fluorescence polarization of the fluorophore. As shown in **Figure 2** and summarized in **Table 1**, all of the analogs had sub-micromolar affinities for the receptor. The only two compounds showing significantly different affinity for the receptor were the compounds with short extensions from the side chain terminating in primary amines (**5** and **7**). This could perhaps be due to some somewhat unfavorable interaction between the polar amine group and some nonpolar residues at the outer boundary of the binding pocket. A comparison of compounds **5** and **7** to compound **9** seems to indicate that pushing the primary amine further out of the binding pocket appears to be sufficient to overcome this unfavorable interaction.

Cell-based reporter assays

The ability of the compounds to modulate estrogen receptor-mediated gene transcription was tested using a luciferase reporter gene assay. The ER-negative HeLa cervical cell line was transiently transfected with a plasmid expressing human ERα and a plasmid containing the luciferase gene under the control of the vitellogenin promoter. This promoter contains two consensus estrogen receptor binding sites and is activated strongly in the presence of ER and estradiol. None of the compounds showed any agonist activity (data not shown), so antagonist activity was determined by performing competition assays in the presence of 10 nM estradiol. As shown in **Figure 3** and summarized in **Table 1**, the compounds were all antagonists of estradiol-induced ER activation at the vitellogenin promoter at relatively low concentrations. Although the variability between assays is much greater with cell-based assays than with the in vitro binding assay, compound **5** showed significant decrease in antagonist potency compared to the other compounds.

Whether this decrease is due to weaker binding affinity for the receptor or diminished cell uptake is unknown. Overall, however, all of the tamoxifen analogs all inhibited ER-mediated transcriptions at concentrations that are low enough to allow for future derivatization studies.

Conclusion

In summary, a novel set of tamoxifen analogs has been made using a relatively simple synthetic scheme. Receptor affinity assays and reporter gene assays indicate that many of the analogs have potencies similar to tamoxifen and would make suitable analogs to conjugate to other moieties in order to study roles of the different pathways leading to estrogen receptor-mediated transcriptional regulation. These moieties will include fluorescent molecules that will allow for the visualization of binding either inside the cell or on the cell surface. The analogs will also be conjugated to cell-impermeable polyacrylate polymers that should allow for selective targeting of membrane-initiated responses of estrogen receptor. It is envisioned that these tools will help elucidate the pleiotropic behavior of tamoxifen and could be used in the future to help engineer novel transcription factors that could either activate or repress the transcription of specific genes.

Materials and Methods

General methods

All reagents were purchased from Sigma-Aldrich. The expression plasmids used in this study, pSG5-ERα and ERE-luciferase were generously provided by Thomas Scanlan (UCSF) and have been described elsewhere [13, 14]. The ERE-driven luciferase reporter gene consists of two repeats of the upstream region of the vitellogenin ERE promoter from –331 to –289, followed by region –109 to +45 of the thymilidate kinase upstream region and the luciferase gene. Proton and ^{13}C nuclear magnetic resonance spectra (1H NMR, 13C NMR) were obtained on a Bruker ARX300 (300 MHz) instrument; 1H NMR chemical shifts are reported as δ values in parts per million (ppm) downfield from internal tetramethylsilane. 13C NMR chemical shifts are reported as δ values with reference to the solvent peak. Mass spectrometry (MS) and NMR instruments were provided by the Shared Resource center of the Purdue Cancer Center.

Synthesis of tamoxifen analogs

***E* and *Z* 4-{1-[4-(2-Bromo-ethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (4)** Diphenol (**3**) (0.5 g, 1.59 mmol) [11] was dissolved in DMF (10 mL) and then cesium carbonate (2.07 g, 6.4 mmol, 4 equiv.) was added and the solution was heated at 60 ˚C for 15 minutes. 1,2 dibromoethane (0.5 mL, 5.7 mmol, 4.5 equiv.) was then added all at once and the reaction was allowed to stir for 16 hr. at 60 ˚C. 30 mL of water was then added to the reaction mixture and the compounds were extracted with ethyl acetate twice. The organic layer was washed with brine, dried with magnesium sulfate and then the solvent was evaporated under reduced pressure. Purification by flash silica gel chromatography using 30% ethyl acetate in hexane as the eluent provided 0.25 g of desired product (0.59 mmol, 37% yield) as a mixture of interconverting E and Z isomers. 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.94 (2H, d) δ 6.83 (1H, dd) δ 6.78 (1H, d) δ 6.62 (1H, d) δ 6.56 (1H, d) δ 4.69 (1H, t) δ 4.57 (1H, t) δ 4.12 (1H, t) δ 4.01 (1H, t) δ 3.10 (2H, q) δ 1.77 (3H, t); 13C NMR (300 MHz) (CDCl3) δ 157.18, δ 153.83, δ 142.95, δ 141.704, δ 138.01, δ 137.48, δ 136.29, δ 132,55, δ 131.15, δ 130.13, δ 128.24, δ 126.42, δ 115.43, δ 114.75, δ 114.00, δ 68.30, δ 29.68, δ 14.06. MS (CI) m/z 423/425 $(M + H)^+$;

General synthesis of amine analogs The bromide (**5**) (50 mg, 0.12 mmol) was dissolved in THF (2 mL) and 0.5 g of the appropriate diamine (as described below) was then added and the solution was heated at 60 ˚C for 12 hr. in a sealed tube. The solvent was then removed evaporated under reduced pressure and then purification by silica gel flash chromatography using 5.5/4/0.5 $CHCl_3/CH_3OH/NH_4OH$ as the eluent provided the product as a mixture of interconverting *E* and *Z* isomers. Below is information for each compound:

***E* and *Z* 4-{1-[4-(2-Aminoethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (5)** NH_4OH was used as the amine and 43 mg of purified product was isolated (0.11 mmol, 92% yield). 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (1H, t) δ 3.49 (1H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t);. MS (CI) m/z 360 (M+H);

***E* & *Z* 4-{1-[4-(2-Methylaminoethoxy)-phenyl]-2-phenyl-but-1-enyl}-phenol (6)** 2 M methylamine in THF was used as the amine and 35 mg of purified product was isolated (0.094 mmol, 78% yield). 1H NMR (300 MHz) (CDCl3) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 5.76 (2H, s) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (1H, t) δ 3.49 (1H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t); 13C NMR (300 MHz) (CDCl3) δ 157.61, δ 156.77, δ 156.26, δ 155.34, δ 143.18, δ 141.20, δ 138.43, δ 137.28, δ 136.78, δ 135.47, δ 135.10, δ 132.43, δ 131.13, δ 130,16, δ 128.26, δ 126.27, δ 115.71, δ 115.04, δ 114.40, δ 113.65, δ 66.56, δ 50.81, δ 36.14, δ 29.50, δ 14.11. MS (CI) m/z 374 (M+H);

***E* and *Z* 4-(1-{4-[2-(2-Aminoethylamino)-ethoxy]-phenyl}-2-phenyl-but-1-enyl)-phenol (7)** Ethylenediamine was used as the amine and 32 mg of purified product was isolated (0.087 mmol, 73% yield). 1H NMR (300 MHz) (CD_3OD) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.51 (1H, t) δ 4.37 (1H, t) δ 3.58 (3H, t) δ 3.49 (3H, t) δ 3.12 (5H, m) δ 2.02 (1H, s) δ 1.76 (3H, t); 13C NMR (300 MHz) (CD_3OD) δ 159.4, δ 158.5, δ 157.9, δ 157.0, δ 144.6, δ 142.4, δ 142.2 δ 140.2, δ 138.43, δ 137.6, δ 136.3, δ 133.47, δ 132.43, δ 131.13, δ 130,16, δ 128.26, δ 126.27, δ 115.71, δ 115.04, δ 114.40, δ 113.65, δ 66.56, δ 42.13, δ 31.2, δ 29.50, δ 14.11. MS (CI) m/z 403 (M+H);

***E* and *Z* 4-[1-(4-{2-[Methyl-(2-methylaminoethyl)-amino]-ethoxy}-phenyl)-2-phenyl-but-1-enyl]-phenol (8)** N,N' dimethylethylenediamine was used as the amine and 15 mg of purified product was isolated (0.035 mmol, 29% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 4.37 (1H, t) δ 4.12 (3H, t) δ 3.95 (3H, t) δ 3.6 (5H, m) δ 2.58 (3H, s), δ 2.50 (3H,s), δ 2.02 (1H, s) δ 1.76 (3H, t)

E and Z 4-(1-{4-[2-(6-Amino-hexylamino)ethoxy]-phenyl}-2-phenyl-but-1-enyl)-phenol (9) 1,6-diaminohexane was used as the amine and 40 mg of purified product was isolated (0.092 mmol, 77% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 3.6 (5H, m) δ 2.58 (2H, t), δ 2.50 (2H, t), δ 2.02 (1H, s) δ 1.6 (3H, t), δ 1.3 (8H, m)

***E* and *Z* 4-[1-(4-{2-[Methyl-(6-methylaminohexyl)-amino]-ethoxy}-phenyl)-2-phenyl-but-1-enyl]-phenol (10)** N,N' dimethyl-1,6-diaminohexane was used as the amine and 18 mg of purified product was isolated (0.037 mmol, 31% yield). 1H NMR (300 MHz) ($CDCl_3$) δ 7.15 (7H, m) δ 6.88 (1H, d) δ 6.81 (2H, dd) δ 6.72 (1H, d) δ 6.58 (1H, d) δ 6.52 (1H, d) δ 3.2 (2H, t) δ 3.1 (2H, t), δ 2.55 (2H, t), δ 2.45 (6H, s), δ 2.22 (2H, t) δ 1.6 (3H, m), δ 1.3 (8H, m).

Fluorescence polarization assay.

Fluorescent polarization based competition binding assays were conducted to determine the relative affinity of the 4-hydroxytamoxifen analogs for ERα using a commercially available kit (PanVera Corp., Madison, WI). Briefly, serial dilutions of the different compounds were prepared in ES2 screening buffer (100mM potassium phosphate, pH7.4, 100μg/ml bovine gamma globulin) and 50μl of each concentration was aliquoted into three wells of a black 96 well assay plate. Fifty microliters of a solution containing 20nM recombinant ERα, and 2nM of a proprietary fluorescent ER ligand (Fluormone-ES2) was added to each well. The plate was shaken on a plate mixer and incubated for 2 h in the dark at room temperature. Fluorescence polarization signals were then measured using a Packard Fusion fluorimeter. The data were then fit to a single binding site competition

curve by nonlinear regression analysis (Prism 3 software package). K_i values were determined from the average of 3 different experiments and calculated using a K_D=4 nM for Fluormone binding to ERα.

Cell culture and transient transfection experiments

Cell Culture HeLa cells were obtained from the American Type Culture Collection (ATCC). HeLa cells were maintained in DME media without phenol red (Sigma) supplemented with 4.5 g/L glucose, 0.876 g/L glutamine, 100 mg/L streptomycin sulfate, 100 units/mL of penicillin G and 10% FBS at 37 °C in a air/carbon dioxide (95:5) atmosphere. Transfection assays were run with the same media conditions except the FBS was treated for 24 hours with dextran-coated charcoal.

Transient transfection assays HeLa cells were plated in 24 well plates and grown to approximately 70-80% confluency. Transfections were performed according to the protocol for Lipofectamine 2000® (Invitrogen). In order to normalize for the transfection efficiency in each well, the dual luciferase system was used in which a constitutively expressed, chemically orthogonal luciferase expression vector was also transfected. The total amount of DNA/well for each plasmid was as follows: pSG5-ERα 0.25 μg/well, ERE-luciferase 0.5 μg/well, and *Renilla*-luciferase 0.25 μg/well. The ratio of total DNA/Lipofectamine 2000® ratio was 1:5. After transfection, the plates incubated at 37 ˚C for 6 hours before dosing with drug. All drugs were delivered in DMSO or ethanol and the total concentration of organic solvent in each was 0.1% For competition experiments, the drug was added to media already containing 10 nM estradiol. After 18-24 hours, the cells were lysed and assayed for dual luciferase activity in a TopCount luminometer according to the protocol provided by Promega. The relative light units (RLU) were then calculated by dividing the output of the ERE-driven luciferase in each well by the output of the *Renilla* luciferase. Each drug concentration was tested in triplicate.

Acknowledgements We acknowledge the financial support of the American Cancer Society (IRG-58-006-41A), the Indiana Elk's Charities, the Army Breast Cancer Research Program (BC030507) and the American Association of Colleges of Pharmacy New Investigator Program. J.P.T. was supported by an IRL Fellowship from the National Science Foundation. P.T.R. was supported by a MARC predoctoral fellowship from the National Institutes of General Medical Sciences (F31-GM67595-01)

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Captions

Figure 1. estradiol (**1**) and tamoxifen (**2**)

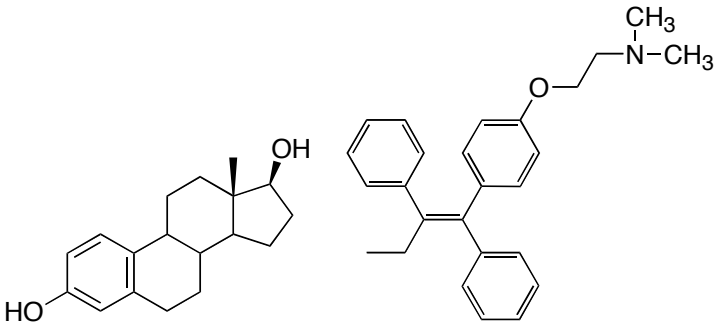
Scheme 1. a.) Cs_2CO_3, DMF, 60 ˚C; 1,2 dibromoethane, 16 hrs. b.) RNHR', THF, 60 ˚C, sealed tube, 12 hrs.

Figure 2. Relative ER binding affinity of tamoxifen analogs **7-10**. The ability of various concentrations of different compounds to displace a synthetic fluorescent estrogen from recombinant preparations of ERα was evaluated as described in the material and methods section. 100 represents no displacement of fluorescent ligand, zero represents total displacement. Each point represents the mean and standard error of the mean of 3 different samples. The lines represent the best fit to a single binding site competition model. Dashed lines represent the fit for the methylated compounds.

Figure 3. Competition of the compounds **7-10** versus 10 nM estradiol in transient transfection assay of HeLa cells with ERα and the vitellogenin A2 ERE-tk driven luciferase reporter gene. Curve represents the best fit to a single-site competition binding model. 100% activation represents the activation with 10 nM estradiol alone. Each point represents the mean and standard error of the mean of 3 different samples. Lines represent the best fit to a single binding site competition model. Dashed lines represent the fit for the methylated compounds.

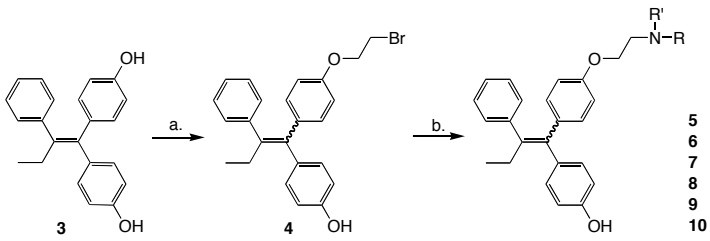
Table 1. Summary of Ki values for compounds calculated from the receptor competition experiments and IC50 values vs. 10 nM estradiol calculated from the reporter gene assays.

Figure 1



(**1**) (**2**)

Scheme 1.



	R	R'
5	H	H
6	CH_3	H
7	H	$(CH_2)_2NH_2$
8	CH_3	$(CH_2)_2NHCH_3$
9	H	$(CH_2)_6NH_2$
10	CH_3	$(CH_2)_6NHCH_3$

Figure 2

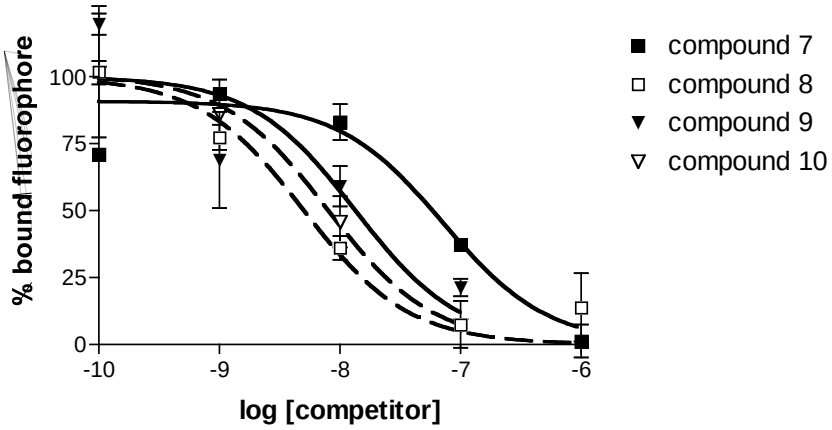

% bound fluorophore
100
75
50
25
0
-10
-9
-8
-7
-6
log [competitor]
compound 7
compound 8
compound 9
compound 10

Figure 3

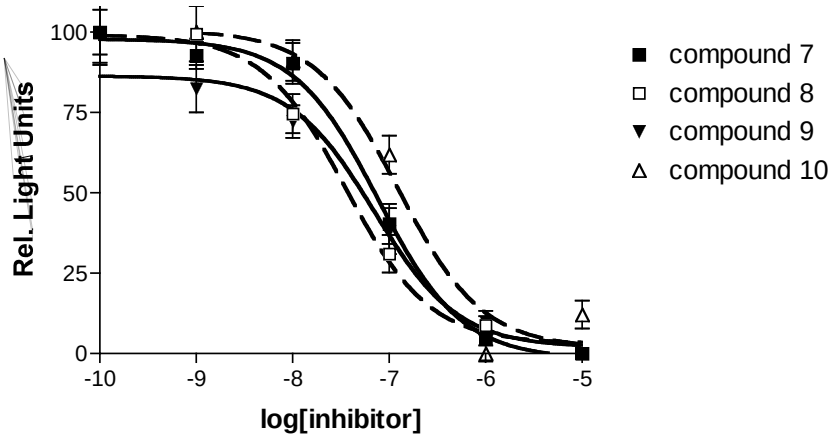

Rel. Light Units
100
75
50
25
0
-10
-9
-8
-7
-6
-5
log[inhibitor]
compound 7
compound 8
compound 9
compound 10

Table 1

compound	Ki (nM)	IC50 (nM)
estradiol **(1)**	6.3 ± 0.2	N.D.
5	48 ±5	800 ± 400
6	8.5 ± 3.9	40 ± 10
7	32 ± 10	150 ± 50
8	3.4 ± 2.1	39 ± 12
9	9.8 ± 6.2	85 ± 55
10	6.2 ± 4.6	126 ± 33

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PRECLINICAL STUDY

Characterization of molecular and structural determinants of selective estrogen receptor downregulators

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Received: 7 June 2006 / Accepted: 20 July 2006

Abstract Antiestrogens used for breast cancer therapy can be categorized into two classes that differ in their effect on estrogen receptor (ER) alpha stability. The selective estrogen receptor modulators (SERMs) stabilize ER alpha and the selective estrogen receptor downregulators (SERDs) cause a decrease in cellular ER alpha levels. A clinically relevant antiestrogen, GW7604, appears to work through a SERD-like mechanism, despite sharing the same molecular scaffold as 4-hydroxytamoxifen, a SERM. In order to investigate potential structural features of GW7604 responsible for SERD activity, GW7604 and two analogs were synthesized using a new, improved synthetic route and tested for their effects on ER alpha function and cell proliferation. The two analogs, which have an acrylamide or a methyl vinyl ketone replacing the acrylic acid group of GW7604, display lower binding affinity for ER alpha than GW7604, but show similar antagonism of estradiol-induced activation of ER alpha-mediated transcription as GW7604 and inhibit estradiol-induced proliferation of the MCF-7 cell line with a similar potency as GW7604. Unlike GW7604, neither analog has a significant effect on cellular ER alpha levels, suggesting that the carboxylate is a key determinant in GW7604 action and, for the first time, showing that this group is responsible for inducing ER alpha degradation in breast cancer cells.

Keywords Antiestrogen · GW5638 · GW7604 · Estrogen receptor degradation · Selective estrogen receptor downregulator · Selective estrogen receptor modulator · Tamoxifen

Introduction

Tamoxifen (Fig. 1) antiestrogen therapy is one of the first and most effective treatments for the treatment and prevention of estrogen receptor (ER) positive breast cancer. Another antiestrogen, fulvestrant, has recently entered the clinic in the United States (Fig. 1). Dramatic differences between tamoxifen and fulvestrant at both the cellular and structural level have been demonstrated [1]. Tamoxifen, which belongs to a class of compounds known as selective estrogen receptor modulators (SERMs), stabilizes ER alpha and causes a slight increase in receptor levels; in contrast, fulvestrant causes rapid ER alpha degradation, leading some to classify compounds such as fulvestrant as selective estrogen receptor downregulators (SERDs) [2]. These differences in mechanism of action of SERMs and SERDs appear to extend to the mechanisms of resistance to these compounds [3]. Many tumors that acquire tamoxifen resistance but remain ER positive are still sensitive to fulvestrant. As a result, there is much interest in finding other compounds with SERD-like mechanisms

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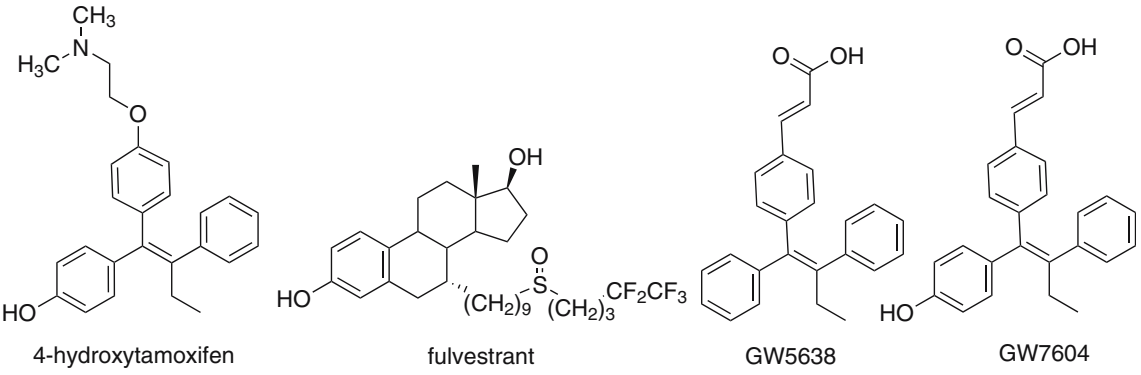

Fig. 1 4-hydroxytamoxifen, fulvestrant, GW5638 and GW7604

and understanding how those compounds cause estrogen receptor degradation.

Two antiestrogens under clinical investigation, GW5638 and its hydroxylated metabolite GW7604 (Fig. 1), have been identified to possess SERD activity similar to fulvestrant and the ability to inhibit the growth of tamoxifen-resistant breast tumors [4, 5]. In contrast to fulvestrant, GW7604 possesses a nonsteroidal structure with a triphenylethylethylene core similar to 4-hydroxytamoxifen. However, GW7604 contains an acrylic acid side chain extending from the triphenylethylethylene core, instead of the basic amine-containing side chain of 4-hydroxytamoxifen (Fig. 1). Exploring the relative importance of the acrylic acid side chain in the overall SERD profile of the GW7604 compound could give insight into the structural determinants for distinguishing SERM and SERD mechanisms and lead to the design of improved antiestrogen therapies for tamoxifen-resistant tumors. In this report, we describe the synthesis and characterization of two new GW7604 analogs and demonstrate that although the carboxylate of GW7604 is essential for eliciting the degradation of ER alpha, this group is not essential for inhibiting the proliferation of breast cancer cells.

Methods

Synthesis of 7604 analogs

The detailed synthetic procedures and characterization for the compounds used in this work can be found in the supplementary material.

ER alpha binding assay

Commercially available fluorescent polarization based competition binding assays (Invitrogen) were used to determine the relative affinity of the GW7604 analogs. Briefly, serial dilutions of the different compounds were prepared in ES2 screening buffer (100 mM potassium phosphate, pH7.4, 100 μg/ml bovine gamma globulin) and 50 μl of each concentration was aliquoted into three wells of a black 96 well assay plate. Fifty microliters of a solution containing 20 nM recombinant ER alpha and 2 nM of a proprietary fluorescent ER ligand (Fluormone-ES2) were added to each well. The plate was incubated for 2 h at room temperature (in the dark with shaking). Fluorescence polarization signals were then measured using a Packard Fusion fluorimeter. The data were fit to a single binding site competition curve by nonlinear regression analysis (Prism 4 software package, Graphpad software). K_i values were determined from the average of 3 different experiments and calculated using a K_D = 4 nM for Fluormone binding to ER alpha.

Transcriptional reporter assays

MCF7/ERE-Luc cells, derived from MCF7 cells stably transfected with a luciferase report construct driven by the estrogen responsive element in *pS2* promoter (ERE-pS2-Luc) [6], were seeded in steroid-free medium for 3 days prior to drug treatment. Cell lysates were prepared with passive lysis buffer (Promega Corp., Madison, WI) and luciferase activity determined using the Luciferase Assay System (Promega). Luciferase activity was normalized against total cellular protein and expressed as the mean unit/mg protein ± SE of three independent experiments.

MCF7 proliferation assays

MCF7 cells (2000/well) were plated in 96-well dishes in steroid-free medium and treated with various doses of

drugs. Cell numbers were determined by MTT assay after 3, 6, 9, and 12 days of drug treatment.

ER alpha stability assays

MCF7 cells (5×10^5/dish) were plated in 60-mm dishes in steroid-free medium for 3 days prior to drug exposure. Whole cell extracts were prepared by suspending cells in 0.1 ml of lysis buffer (62 mM Tris, pH 6.8, 2% sodium dodecyl sulfate; 10% glycerol; 10 µl protease inhibitor cocktail set III). After sonication (3×10 sec), insoluble material was removed by centrifugation (15 min at 12,000 *g*), and protein concentration in the supernatant was determined using the Bio-Rad Laboratories, Inc. protein assay kit. The protein extracts were mixed with 1/4 vol of 5× electrophoresis sample buffer and boiled for 5 min at 90 C. Protein extract (50 µg per lane) was then fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies. Primary antibody was detected by horseradish peroxidase-conjugated second antibody and visualized using enhanced SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). The band density of exposed films was evaluated with ImageJ software (http://rsb.info.nih.gov/ij/).

Results

Design and Synthesis of GW7604 Analogs

Although GW5638 and its 4-hydroxylated analog GW7604 share many structural similarities with tamoxifen and 4-hydroxytamoxifen, they appear to modulate ER alpha activity by different mechanisms. Structural information garnered from a crystallographic study with GW5638 bound to the ligand binding domain (LBD) of ER alpha suggests that the acrylic acid side chain of GW5638 induces helix 12 of the LBD to adopt a conformation distinct from the conformation induced by 4-hydroxytamoxifen [7]. The carboxylic acid of the acrylic acid side chain of GW5638 appears to be involved in hydrogen bonds with a bound water molecule and the side chain of aspartate 351 and the backbone amide of leucine 536. The acrylic acid side chain of GW5638 has been shown previously to be important in the overall function of the compound—GW5638 analogs possessing an acrylamide side chain showed equivalent uterotrophic activity as tamoxifen in immature rats compared to the non-uterotrophic activity of 5638 [8]. Furthermore, modification of the acrylic acid side chain to either an acrylamide or a vinyl methyl ketone altered the activity of ER alpha at a specific AP-1 regulated promoter [9].

The unique effects of the acrylamide and methyl vinyl ketone analogs of GW5638, combined with the fact that the 4-hydroxylated compound GW7604 showed significantly more potent activity than GW5638, led to the design of a new synthesis to make a novel acrylamide derivative and remake the methyl vinyl ketone derivative of GW7604. The previously reported synthesis of GW7604 and its methyl vinyl ketone derivative was found to be inadequate for the needs of this study due to two very poor yielding steps that were intractable to optimization—the protection of the phenol as a tetrahydropyran acetal and the formation of a vinyl bromide intermediate. As a result, a new synthesis was designed that relied on a high yielding Friedel–Crafts acylation and Grignard coupling reaction to generate the triphenylethylene core (Fig. 2) [10, 11]. The dehydration generated both stereoisomers of the double bond, but after deprotection of the phenol, the double bond of the triphenylethylene interconverted readily at room temperature, as had been shown previously [9]. That work also showed that only one isomer of GW5638 had biological activity, so it is highly likely that ER alpha only bound to the *E* isomer of these GW7604 analogs. The remainder of the synthesis followed previously reported work to readily generate GW7604 and 7604-ket and a novel analog, 7604-NH2.

Estrogen receptor binding assays

After synthesizing the compounds, we first determined whether the modifications altered the binding affinity to ER alpha. Using a fluorescence polarization-based competition assay with purified full-length ER alpha, the K_i values were determined to be 27 ± 10 nM for GW7604, 240 ± 35 nM for 7604-NH2 and 210 ± 30 nM for 7604-ket (Fig. 3). The K_i determined for GW7604 and 7604-ket are consistent with previous studies [9]. The binding data suggest that although altering the carboxylic acid to either a carboxamide or a methyl ketone reduces the affinity of the ligand for ER alpha significantly ($P < 0.01$, one-way ANOVA test with Dunnett's post-test), the compounds possess sufficient receptor affinity to perform cell-based experiments.

Estrogen receptor transcriptional activity

After testing the binding affinity, we examined the ability of these compounds to modulate ER alpha

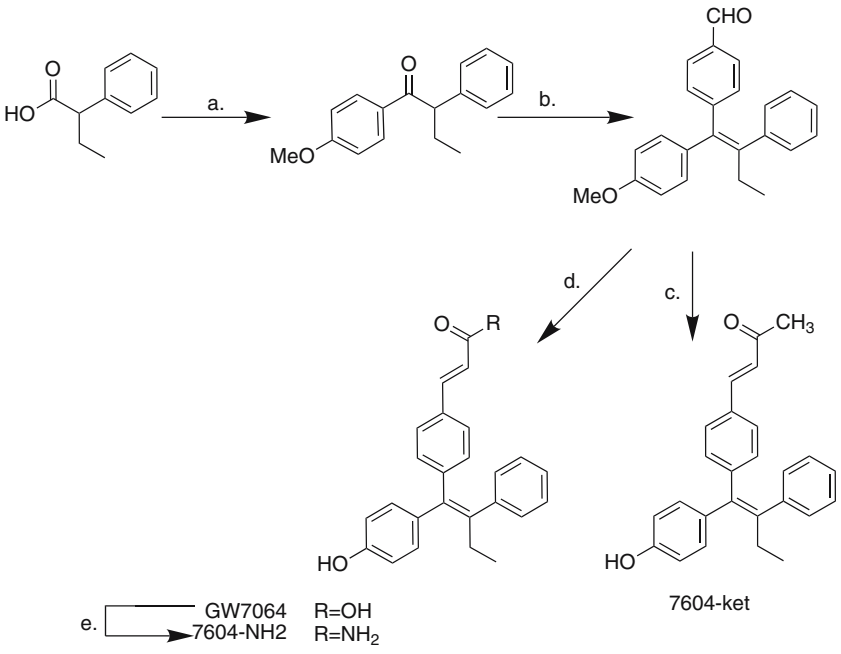

Fig. 2 Synthetic scheme for the preparation of 7604 analogs. (**a**) 2-phenylbutyric acid, trifluoroacetic acid anhydride, phosphoric acid, anisole, 10 °C, 100% yield, (**b**) (i)., THF, magnesium, 4-bromobenzaldehyde diethyl acetal; H_3O^+(ii). HCl, ethanol, reflux, 76% yield. (**c**) (i). diethyl (2-oxopropyl)phosphonate, potassium bis(trimethylsilyl)amide, THF, –78 °C to room temp. (ii). BBr_3, CH_2Cl_2, 0 °C, 54% yield. (**d**) (i).trimethylphosphonoacetate, potassium bis(trimethylsilyl)amide, THF, –78 °C to room temp. (ii). KOH, EtOH/THF, reflux (iii). BBr_3, CH_2Cl_2, 0 °C, 37% yield. (**e**) EDC, HOBT, Et_3N, NH_4OH, DMF, 80% yield

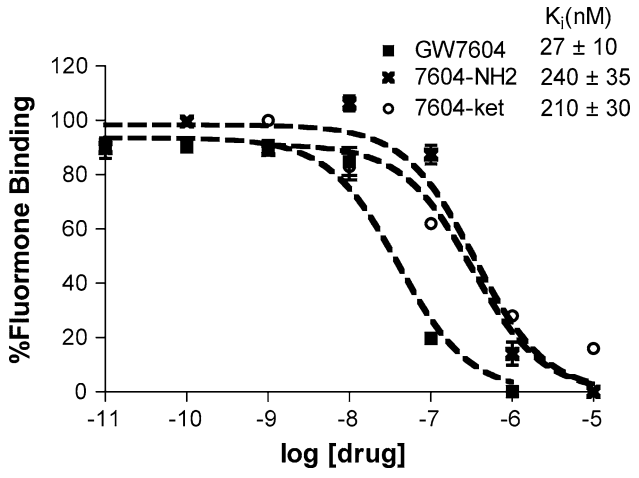

Fig. 3 Binding of 7604 analogs to ER alpha 2 nM of Fluormone ES2 was incubated with recombinant ER alpha in the presence of various concentrations of 7604 analogs and the extent of displacement of fluorescent ligand measured using fluorescence polarization

transcriptional activity inside cells by using MCF7 breast cancer cells stably transfected with an ERE-pS2-Luc construct [6]. All three GW7604 compounds acted as antagonists but showed different potencies, depending on whether hormone was present or absent. In the absence of E2, inhibition of basal reporter gene activity by 7604-NH2 was greater than GW7604 or 7604-ket. However, GW7604 displayed greater inhibition of E2-induced reporter gene activity than 7604-NH2 and 7604-ket (Fig. 4). Consistent with the ER alpha receptor binding data, both 7604-NH2 and 7604-ket were significantly less potent than GW7604 at antagonizing E2-induced transcription of the stably integrated ERE-pS2-Luc reporter.

Receptor stability

One of the most interesting properties of GW7604 is its ability to induce ER alpha degradation after binding to the receptor [12]. In order to determine whether the carboxylic acid group was important in inducing degradation, ER alpha levels were measured in MCF7 cells after treatment with the various analogs. As shown in Fig. 5, GW7604 induced ERα degradation in a dose dependent manner, but the acrylamide and methyl vinyl ketone analogs did not induce degradation to nearly the same extent. Even with extended incubation times, the extent of ER alpha degradation induced by the acrylamide and the methyl vinyl ketone was much less than the degradation induced by GW7604. Taken together, these observations indicate that the carboxylate moiety of GW7604 is essential for its selective estrogen receptor degradation properties.

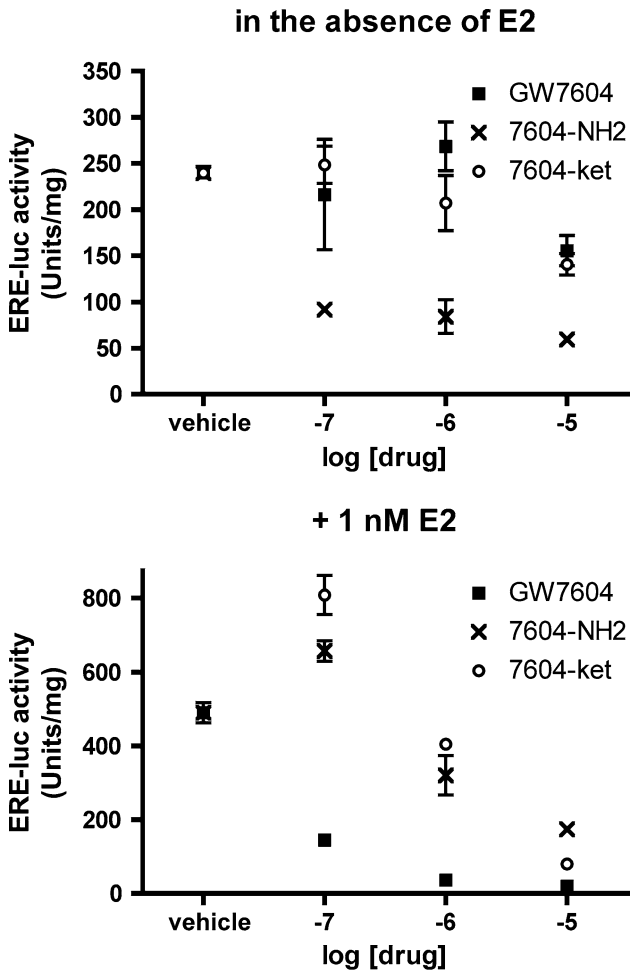

Fig. 4 Effect of 7604 analogs on ER alpha transcription activity. MCF7/ERE-Luc cells were seeded in hormone-free medium for three days, then treated with 7604 analogs as indicated, in the absence or presence of 1 nM E2. Luciferase activity was examined at 24 h after drug treatment. Luciferase activities are normalized against total cellular protein and expressed as the mean units/mg protein ± SE of three independent experiments

Proliferation assays

Because the extent of ER alpha degradation induced by the two GW7604 analogs was not significant, it was unclear whether these compounds would still inhibit estrogen-induced proliferation of breast cancer cells. A standard MTT cell proliferation was performed using MCF-7 cells grown in hormone free media (Fig. 6). In the absence of estradiol, GW7604 and 7604-ket, but not 7604-NH2, significantly inhibited basal cell growth at high doses (10^{-7}–10^{-6} M, $P < 0.05$ versus vehicle, student's *t*-test). In the presence of 1 nM estradiol, however, inhibition of cell growth was observed for all three compounds at approximately the same concentrations, suggesting that the two 7604 analogs act as antiestrogens in the breast, even though they do not induce ER alpha degradation in a fashion similar to GW7604.

Discussion

Selective estrogen receptor degradation represents an emerging, clinically validated paradigm in designing antiestrogen treatments for breast cancer. One major benefit to using a SERD such as fulvestrant compared to using a SERM such as tamoxifen is that SERDs have been found to still effectively treat some ER alpha-positive, tamoxifen-resistant breast cancers [13]. Thus, compounds that induce ER alpha degradation may be used to extend the period of time that breast cancer patients can be treated successfully with antiestrogen therapies, presumably by using different SERMs, aromatase inhibitors and SERDs in succession [14].

While fulvestrant is considered an effective therapeutic agent for treatment of advanced breast cancer [1, 13], a major problem at the current time is poor bioavailability, thereby requiring monthly intramuscular injections for drug delivery. In addition, the synthesis of fulvestrant is lengthy and difficult to modify in order to study structure-activity relationships related to the ability of the drug to induce ER alpha degradation. Due to the difficulty of working with fulvestrant, the finding that GW7604 induced ER alpha degradation provided an excellent opportunity to study the molecular mechanisms of SERD activity.

Even though both fulvestrant and GW7604 induce ER alpha degradation, these compounds are significantly different molecules. Fulvestrant is a steroidal compound with an extremely long, flexible extending side chain, whereas GW7604 has a rigid, nonsteroidal structure and an extending side chain that terminates in a carboxylic acid—a rarity in compounds that target the ER alpha. The fact that both of these compounds could induce ER alpha degradation was initially puzzling. However, the crystal structures of GW5638 and fulvestrant bound to the ER alpha ligand binding domain (LBD) were recently reported [7, 15], revealing that receptor conformations induced by both compounds exposed hydrophobic residues, which are normally ''packed'' inside the LBD, to the surrounding solvent. Exposed hydrophobic patches on the protein surface are known targeting signals for protein degradation [16], and fulvestrant and GW5638 induce this repositioning of hydrophobic residues through different mechanisms. The long side chain of fulvestrant blocks any interaction of helix 12 with the rest of the LBD, resulting in exposure of the hydrophobic core of the receptor binding pocket to solvent. In contrast, GW5638 causes less disruption of helix 12 than fulvestrant, but the carboxylic acid of GW5638 forms hydrogen bonds with the amide backbone of Leu536

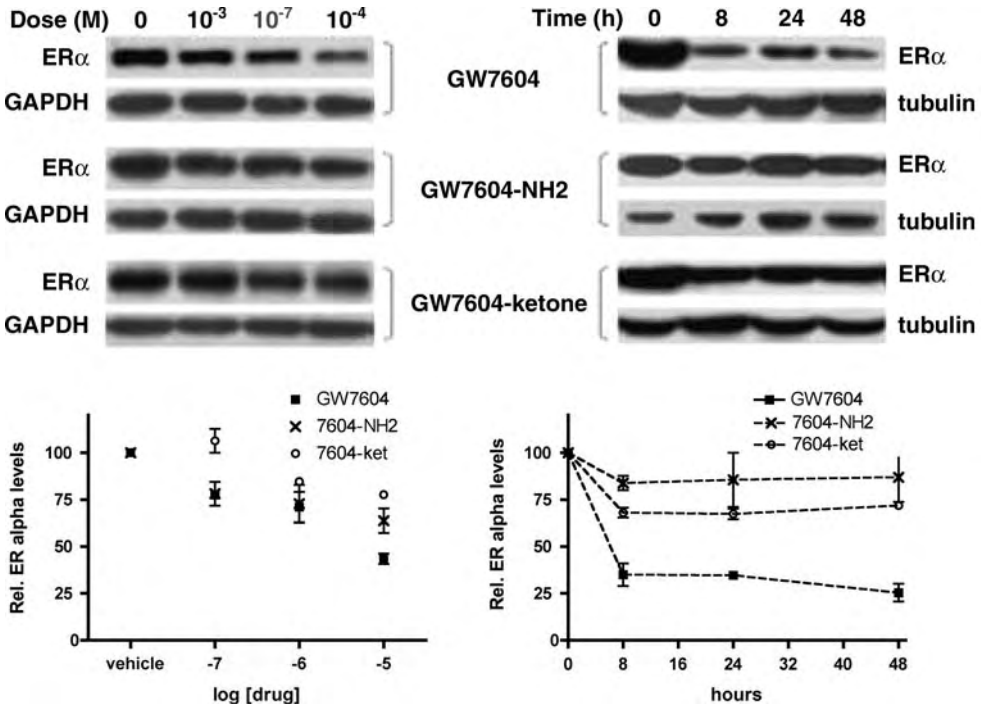

Fig. 5 Effects of 7604 analogs on ER alpha stability. MCF7 cells were seeded in hormone-free medium for three days, then treated with 7604 analogs for various times as indicated. ER alpha levels in whole cell extracts were determined by immunoblotting with anti-ERα antibody. GAPDH or tubulin was used as the loading control. Representative results of experiments performed in duplicate are shown. Relative ER alpha levels (versus untreated cells) are shown in the corresponding histogram

and Tyr537, tethering that region of helix 12 closer to the ligand binding pocket and distorting the positioning of the other hydrophobic residues of helix 12 (Fig. 7). This key interaction between the carboxylic acid and the residues of helix 12 led us to explore the effect of changing that carboxylic acid on the function of GW7604.

The analysis of the GW5638-ER alpha LBD structure suggests that the acrylic acid group on GW5638 is protonated. If this is true, then converting the carboxylic acid of GW7604 to a carboxamide is a fairly conservative change. The carboxamide is not exactly isosteric with the carboxylic acid and the protons on the carboxamide are much less acidic, but the carboxamide is still capable of hydrogen bonding and could potentially hold the helix 12 backbone in the same degradation-inducing conformation when bound in the binding site. Converting the carboxylic acid to a methyl ketone would generate a compound capable of fitting into the binding pocket but unable to engage in the same number of hydrogen bonds as the carboxylic acid of GW7604. The ketone would likely not be able to maintain the necessary contacts with backbone amide hydrogens in helix 12 to induce degradation.

Making conservative changes in the carboxylic acid moiety proved to be deleterious when the ER alpha binding affinity of the two analogs was measured. Both analogs bound to the receptor with lower affinity but the equilibrium dissociation constants were still in the nanomolar range, suggesting that the modifications were still mostly compatible with the binding pocket. Both analogs also inhibited ER alpha mediated transcription from an ERE-controlled promoter, another indication that the compounds were able to disrupt the normal packing of helix 12 to form the coactivator binding pocket. Even though the two analogs do show some differences with GW7604 from the viewpoint of binding and transcriptional regulation, the two analogs differed significantly from GW7604 in terms of effects on ER alpha stability. GW7604 induced ER alpha degradation in a dose dependent and time dependent manner, whereas the two analogs had minimal effects on ER alpha levels. Overall, this difference did not have a significant effect on the ability of the two analogs to inhibit estradiol-induced MCF7 proliferation, as both GW7604-ket and 7604-NH2 inhibited cell growth to nearly the same extent as GW7604. For both the ERE transcriptional assays and the cell proliferation assays, the different effects seen for the 3 compounds in the absence of estradiol are not easily rationalized, but we speculate that these differences reflect the ability of the compounds to induce distinctive conformational changes in ER alpha that affect basal levels of activity.

Ultimately, these results suggest that modification of the carboxylate moiety of GW7604 converts the mechanism of action from a SERD-like mechanism found with fulvestrant to a SERM-like mechanism found with tamoxifen and raloxifene. Comparing the binding modes of the side chain extension of GW5638

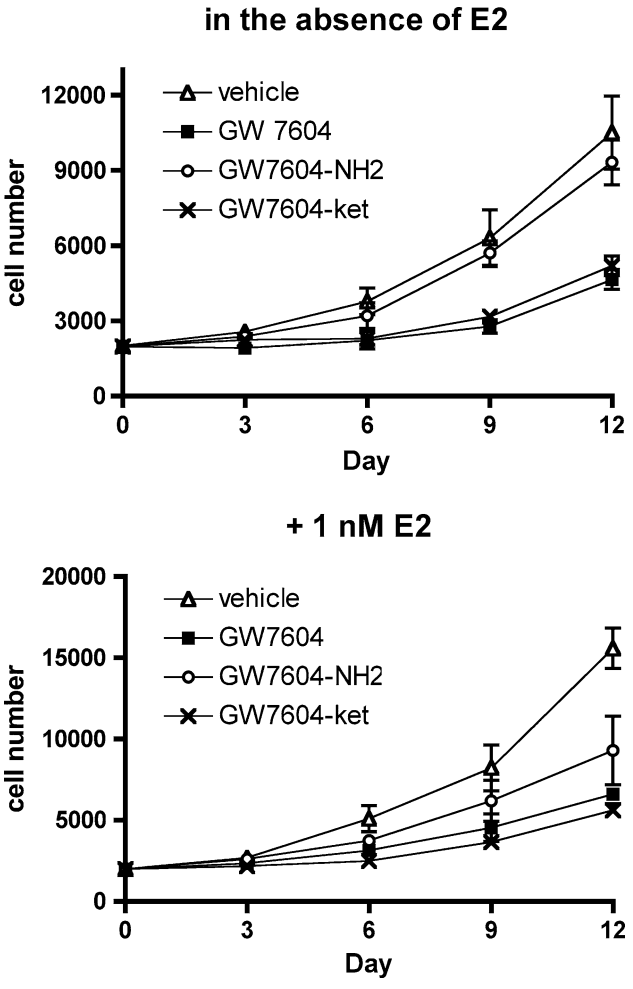

Fig. 6 Effect of 7604 Analogs on MCF7 cell growth. MCF7 cells were seeded in hormone-free medium and treated with 7604 analogs as indicated, in the absence or presence of 1 nM E2. Seven days after treatment, cell number was determined by MTT assay. Experiments were performed twice in triplicate

and 4-hydroxtamoxifen with ER alpha (Fig. 7) shows that GW5638 is able to make hydrogen bond contacts with the helix 12 backbone protons whereas 4-hydroxytamoxifen does not. It is likely that the acrylamide and methyl vinyl ketone analogs are also unable to make the necessary number of hydrogen bonds to the helix 12 backbone, either due to steric effects or lack of appropriate hydrogen bond donor or acceptor groups. Because GW7604-ket and 7604-NH2 likely interact with Asp351, helix 12 can still be displaced and antagonize transcription in a manner similar to 4-hydroxytamoxifen, i.e., a more ''SERM-like'' mechanism of action. The analogs do not induce ER alpha degradation, indicating that repositioning of helix 12 into a conformation that exposes hydrophobic residues does not occur.

In conclusion, we have characterized the activity of two new antiestrogens and demonstrated, for the first time using very slight chemical changes, the conversion of an antiestrogenic compound and ''ER downregulator'' into a SERM and ''receptor stabilizer''. The implications of our findings may have clinical significance. Breast tumors that become resistant to one antiestrogen class often maintain sensitivity to another class of antiestrogens. Based on our observations, we suggest that two distinct classes of therapeutics can be derived from one tight binding lead structure. Modifications that allow for additional interactions between the ligand and receptor appear to be key determinants for designing new ER downregulators (i.e. SERDs) with potential clinical use. Such interactions, which also cause a slight unfolding of the LBD, expose hydrophobic residues to solvent. Unfortunately, at this time, there are no general rules for eliciting such

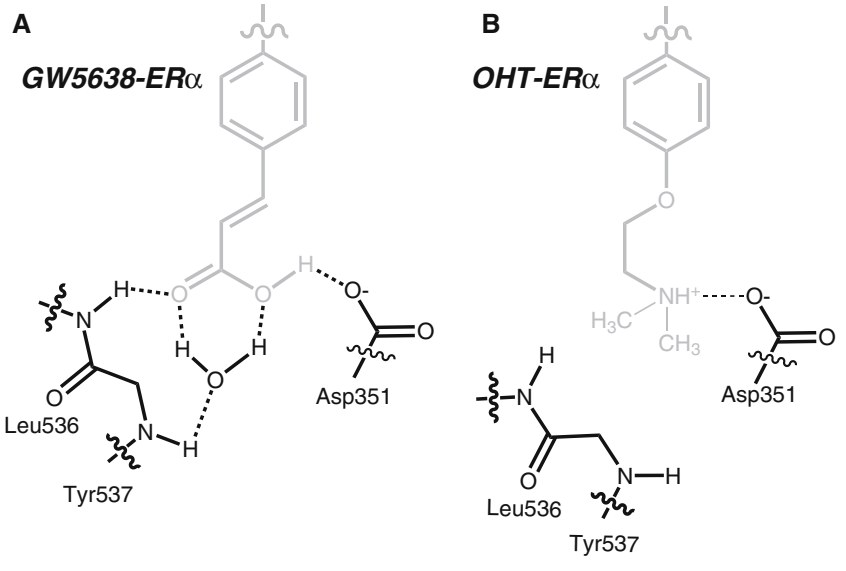

Fig. 7 Binding of GW5638 and 4-hydroxytamoxifen side chains. Cartoon schematic of the interactions between the side chains of the ER alpha ligand binding domain with the side chain extension of (**A**) GW5638 and (**B**) 4-hydroxytamoxifen. Triphenylethylethylene core and side chain residues of Leu537 and Tyr537 are omitted for clarity. Dashed lines represent hydrogen bonds

unfolding, and further study into the mechanistic differences between different types of antiestrogens is needed in order to extend the usefulness of high affinity pharmacophores.

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